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Pseudomonas

Virulence and Gene Regulation

Edited by Juan-Luis Ramos

Pseudomonas

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Edited by Juan-Luis Ramos, *CSIC, Granada, Spain*

Volume 1: Genomics, Life Style and Molecular Architecture
Volume 2: Virulence and Gene Regulation
Volume 3: Biosynthesis of Macromolecules and Molecular Metabolism

Pseudomonas

Volume 2
Virulence and Gene Regulation

Edited by

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PREFACE

The time was ripe for a series of books on *Pseudomonas* to see the light. About 18 years have passed since Jack Sokatch first published his outstanding *The Biology of Pseudomonas* back in 1986. This was followed by two books published by the ASM that contained the presentations of the *Pseudomonas* meetings held in Chicago in 1989 and Trieste in 1991. The earlier volume was edited by Simon Silver, Al Chakrabarty, Barbara Iglewski and Sam Kaplan, and the later by Enrica Galli, Simon Silver and Bernard Witholt.

The present series of books was conceived at a meeting with Kluwer staff members in August 2002 during the XI IUMS conference in Paris. In less than a year a group of outstanding scientists in the field, after devoting much of their valuable time, managed to complete their manuscripts for the three volumes of the series. It has been an honor for me to work with them.

The review process has also been of great importance to ensure the high standard of each chapter. Renowned scientists have participated in the review, correction and editing of the chapters. Their assistance is immensely appreciated. I would like to express my most sincere appreciation to:

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There has been growing interest in pseudomonads and a particular urge to understand the biology underlying the complex metabolism of these ubiquitous microbes. These bacteria are capable of colonizing a wide range of niches, including the soil, the plant rhizosphere and phylosphere, and animal tissues; more recently they have attracted attention because of their capacity to form biofilms, a characteristic with potentially important medical and environmental implications.

There has been an explosion of vital new information about the genus *Pseudomonas*. A rapid search for articles containing the word "Pseudomonas" in the title in the last 10 years produces more than 6,000 articles! Consequently, we cannot cover all possible topics relevant to this genus in just three volumes, although our intention has been to be as thorough as possible. To organize the books, various topics have been grouped under a common heading, although this has some limitations since certain chapters may seem equally appropriate under different headings.

In Volume 1, the first chapter provides clues to the definition of the true *Pseudomonas* genus and gives a historical perspective of research to date. The insights in this chapter will undoubtedly help us to ascribe potential new isolates to this genus. Several recent advances in genomics, plasmids and phage biology, as well as a wealth of useful tools, are grouped under the heading "Genomics." These chapters reveal the basis for diversity within the genus *Pseudomonas* at the molecular level, as well as key structural features. This section also describes a wide range of molecular tools to study gene expression and cloning in *Pseudomonas*, which have also been of great use in the analysis of other organisms. These chapters are therefore of potential interest to colleagues working in other genera in addition to *Pseudomonas*.

The section "Life styles" comprises a series of fascinating chapters that explain where pseudomonads can be found, their role in a particular niche, and how they interact with other members of the ecological community. Volume 1 concludes with a series of chapters on the architecture of *Pseudomonas* which explain the structure of the cell surfaces, their ability to

sense nutrients, and the use of the flagellar system of move toward chemoattractants or away from chemorepellent compounds. The molecular basis of cell architecture is critical to understand life styles, such as adhesion and secretion or extrusion of toxic substances including antibiotics and solvents. In all, I believe that the chapters included in this volume will by themselves constitute a book of general interest for microbiologists working on the biology of *Pseudomonas*.

Volume 2 deals with two important issues: virulence and gene regulation. The chapters under the heading "Virulence" deal with well-established virulence factors which make *Pseudomonas aeruginosa*, an opportunistic pathogen, and *Pseudomonas syringae*, a plant pathogen. Some issues on virulence were dealt with in Volume 1 under the heading "Life styles"; in Volume 2, the aim is to provide insights into the molecular mechanisms of virulence. In phytopathogens, their life style overlaps with some beneficial properties of non-pathogenic soil bacteria, but what actually makes them different is critical to our understanding of the biology of this group of *Pseudomonas*.

An astonishing finding from the analysis of the genomes sequenced to date is the wide battery of regulatory genes found in *Pseudomonas*. Also surprising is the wide range of alternative sigma factors encoded by these bacteria. Many of these factors belong to the ECF family and are involved in iron acquisition. The regulators of *Pseudomonas* can be placed into two broad groups, namely those that belong to the two-component class, in which a sensor protein "senses" the signal and triggers the actual regulator(s), and those of a second group in which sensing and regulatory functions are located in a single polypeptide. Volume 2 contains the most exhaustive and up-to-date compilation available of sigma -70-dependent promoters in the genus *Pseudomonas*.

Volume 3 comprises the sections "Macromolecules," "Alternative respiratory substrates," "Catabolism and biotransformations" and "Secondary metabolism." The chapters in the section titled "Macromolecules" review some of the most complex metabolic pathways in the bacterial kingdom, and deal with the biosynthesis of LPS, fatty acids, alginate, rhamnolipids, cyclic lipopeptides, heme groups and vitamin B12.

Pseudomonads are well known for their extreme nutritional versatility and their ability to produce added-value products from simple, cheap carbon and nitrogen sources. The chapters in Volume 3 deal with the metabolism of certain amino acids and other natural compounds such as alkanes, as well as some xenobiotics and recalcitrant compounds such as aromatics. Some of the enzymatic properties of pseudomonads have been exploited to produce added-value products, which makes non-pathogenic *Pseudomonas* strains of great interest for certain industrial processes. This, in addition to the ability of certain strains to biosynthesize secondary products and influence the life style of certain strains, also makes them of interest for the industry.

In recent years, it has become clearer that pseudomonads are able to colonize anaerobic niches. In these niches, many strains are able to respire alternative electron acceptors, and the process of denitrification is indeed well understood in some strains of the genus *Pseudomonas*. More recently, a strain able to respire nitroorganic compounds has been reported, and recent studies have revealed that this property may be more widespread than was initially thought.

There remains no doubt in my mind that in the next 10 years we will see myriad articles dealing with *Pseudomonas* and shedding further light on our current understanding of the broad group of bacteria in the genus *Pseudomonas* as it is now defined. However, I am confident that this series of books has assembled a significant part of the current knowledge of *Pseudomonas* in the best possible manner. More importantly, I hope it will open new lines of research that will lead to a better understanding of this group of saprophytic microorganisms.

Last but not least, I would like to acknowledge the enthusiasm and assistance of Carmen Lorente in the compilation of the three volumes that constitute the *Pseudomonas* series.

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VIRULENCE

1

VIRULENCE FACTORS IN *PSEUDOMONAS AERUGINOSA*

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1. INTRODUCTION

Despite major recent advances in the study of the virulence of the human opportunistic pathogen *Pseudomonas aeruginosa*, our understanding of the pathogenesis of *P. aeruginosa* infections remains limited. This pathogen causes a wide range of infections in humans including acute localized infections such as urinary tract infections, acute ulcerative keratitis, malignant otitis media, peritonitis, acute ventilator associated pneumonia in endotracheal intubated patients, and burn wound infections, as well as chronic localized infections such as chronic destructive lung infections in cystic fibrosis (CF) patients[192]. In addition, patients with severe underlying diseases reducing physical (burned patients, mechanically ventilated patients) and/or immune defense mechanisms (neutropenia, AIDS patients) are at serious risk for evolution of localized infections toward systemic disease, which is associated with dramatically elevated mortality.

Just as varied as the clinical diseases caused by *P. aeruginosa*, this typical nosocomial pathogen possesses and produces a large variety of both cell-associated and extracellular virulence factors (Figure 1). Evaluations in various animal models of the roles of these different virulence factors have improved our understanding of the pathogenesis of these infections. It is

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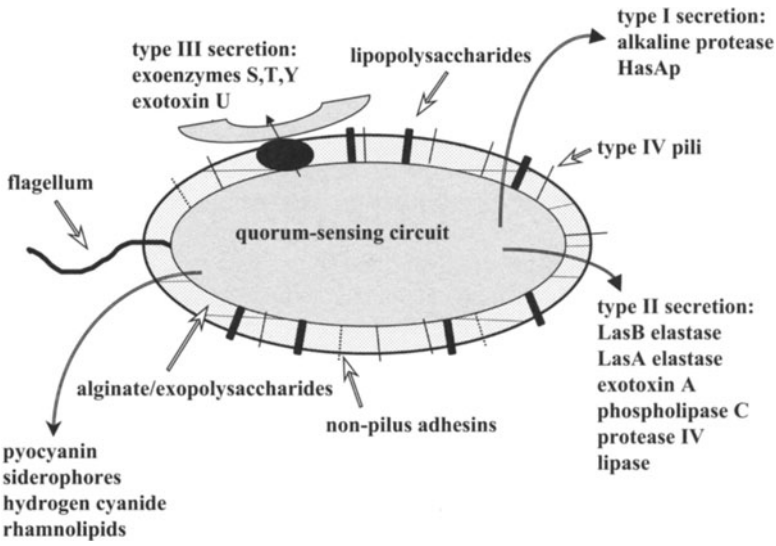

Figure 1. Virulence determinants in *P. aeruginosa*. *P. aeruginosa* has both cell-associated (flagellum, type IV pili, non-pilus adhesins, lipopolysaccharides [LPS]) and extracellular virulence factors (LasB and LasA elastases, exotoxin A [ETA], phospholipase C, protease IV, lipase, exoenzymes S, T and Y, exotoxin U, alginate/exopolysaccharides, pyocyanin, siderophores, hydrogen cyanide and rhamnolipids). The type III secretion apparatus is used to inject proteins into eukaryotic cells (as depicted). The quorum-sensing circuit regulates the production of many extracellular virulence factors.

important to realize that the pathogenesis of *P. aeruginosa* is not related to a single virulence factor, but to the precise and delicate interplay between different factors, leading from efficient colonization and biofilm formation, to tissue necrosis, invasion and dissemination through the vascular system, as well as activation of both local and systemic inflammatory responses.

In this review, we will discuss the various virulence determinants that have been suggested to play a role during the pathogenesis of *P. aeruginosa* infections. Virulence factors can be classified in different ways; based on mechanisms of action, on the infection model where their role has been suggested, or on the pattern of being cell-associated or secreted. We have arbitrarily chosen to classify the various virulence factors according to their physical relation to the bacterium, either cell-associated or secreted into the extracellular space. Extracellular factors are further classified according to their secretion via type I, type II or type III secretion pathways. In addition to typical virulence factors, the quorum-sensing circuit, involved in the regulation of many virulence factors, could itself be viewed as a major virulence

determinant of *P. aeruginosa*. Its role in the pathogenesis of *P. aeruginosa* infections will also be discussed.

2. CELL-ASSOCIATED VIRULENCE FACTORS

2.1. Virulence Factors Promoting Adherence

Frequently, the first step in the pathogenic sequence of *P. aeruginosa* infections is colonization of an epithelial surface. As observed for other bacteria, *P. aeruginosa* uses specific adhesins in order to initiate contact with these biological surfaces. These adhesion factors have been best studied during contact between *P. aeruginosa* with airway epithelial cells leading to the establishment of respiratory tract colonization.

2.1.1. Adherence to Epithelial Cells

P. aeruginosa colonizes the oropharynx of up to 6% of healthy individuals[192]. In contrast, hospitalized patients are at high risk for *P. aeruginosa* colonization with rates approaching 50%[192]. *P. aeruginosa* does not bind easily to uninjured epithelial cell surfaces[45]. The normal respiratory tract is protected from pathogenic bacteria by the mucociliary apparatus. Fibronectin also normally covers epithelial cells and prevents bacterial attachment; however, this protective coating seems to be lost secondary to illness, stress and/or other factors associated with hospitalization. Therefore, adherence of *P. aeruginosa* to epithelial cells is triggered by specific conditions frequently encountered in hospitalized patients. Proteases, present in high levels in the sputum of CF patients, might also be involved in the degradation of fibronectin covering epithelial cells[192]. Cellular injury, secondary to influenza virus challenge of epithelial cells, or occurring during endotracheal intubation, significantly increases the attachment ability of *P. aeruginosa*[201]. This increased binding involves several components of the extracellular matrix that are exposed upon epithelium injury including laminin and type I collagen[42]. It also appears that, whereas *P. aeruginosa* does not interact with soluble plasma fibronectin and cellular fibronectin, it may interact with soluble cellular fibronectin released by epithelial cells which would establish a bridge between bacteria and cell surface receptors[42]. This interaction has been suggested to rely on six fibronectin-binding proteins. One of them might correspond to the major outer membrane protein OprF[204], which has also been suggested to contribute directly to bacterial adherence to epithelial cells[7].

Adherence of non-mucoid *P. aeruginosa* to epithelial cells is mainly mediated by type IV pili which account for 90% of the adherence capacity[44, 98]. Specific molecules on the surface of these structures such as galactose-binding

or mannose-binding lectins (PA-IL and PA-IIL) may act as ligands reacting with complementary sequences on host cells such as the carbohydrate moiety of the glycosphingolipids (GSL) asialo-G_{M1} and G_{M2}, as well as surface-localized proteins of human epithelial cells[42, 98]. Type IV pili binding to asialo-G_{M1} has been suggested to mediate cytoxicity and bacterial internalization[32]. Interestingly, the *P. aeruginosa* lectins PA-IL and PA-IIL have recently been shown to be controlled by quorum sensing which also controls the production of several other important extracellular virulence factors in *P. aeruginosa*[248, 263]. An enzyme with ADP-ribosylation activity called exoenzyme S (ExoS) has also been shown to have GSL binding capacity[195]. The biological relevance of the ExoS-mediated adhesion remains to be established, however.

2.1.2. Adherence to Mucin

P. aeruginosa not only interacts with the epithelium, but also with mucin, the main component of the mucus that forms a viscous gel on the ciliated epithelium of the airways. The normal function of mucin is believed to be trapping and clearing of inhaled particles through the ciliary apparatus. Whereas a bacterial receptor to mucin in normal conditions obviously cannot be considered a virulence factor, this might be different in conditions with impaired mucociliary clearance, such as CF. In these situations, receptors that increase binding to mucin might facilitate colonization of the airways and play an important role in pathogenesis[199]. Interestingly, modifications of mucin, including increased fucosylation and sialylation have been observed in CF patients, and the binding of *P. aeruginosa* to CF mucin is increased, suggesting that qualitative modifications of the carbohydrate chains of CF mucin might increase the affinity for *P. aeruginosa* and enhance colonization[220]. The flagellum, which is primarily responsible for motility, has been shown to function as an adhesin to carbohydrate moieties of the Lewis *x* derivates found at the periphery of human mucin[63, 219]. The fine discrimination associated with mucin recognition by *P. aeruginosa* is highlighted by the finding that different glycoconjugates bearing Lewis *x*, sialyl-Lewis *x* or sulfosialyl-Lewis *x* glycotopes are involved in the binding of the flagellar protein flagellin and either subtypes A or B of the flagellar cap protein FliD[219]. More recently flagellin has also been identified as an adhesin to the epithelial cell associated mucin Muc1[140]. Several other non-pilus adhesins, including the lectin PA-IIL, responsible for the binding to mucin have been described[88]. Recently, the structural basis for PA-IIL mediated adhesion to fucose has been solved by crystallography and has confirmed that antigens of the Lewis *a* series are the preferred ligands for this lectin[160]. As increased fucosylation characterizes CF mucin, these observations open new options for anti-adhesion therapeutic interventions.

2.1.3. Adherence to Abiotic Surfaces

Biofilm formation on abiotic surfaces, such as polyvinyl chloride (PVC), is the initial step of colonization of such surfaces. Mutants defective in flagellum formation have been shown to be severely impaired in their attachment to PVC surfaces and flagella appear to be involved in both motility (to allow close contact with inert surfaces), and in initial cell-surface interactions[169]. In the same study, mutants affected in type IV pili production formed a cellular monolayer on the PVC surface but were unable to form characteristic microcolonies, suggesting that type IV pili play an important role in the formation of these structures. The exopolysaccharide alginate produced by mucoid strains recovered from CF patients also promotes attachment of *P. aeruginosa* to epithelial cells[200].

2.2. Endotoxin

Like other Gram-negative bacteria, *P. aeruginosa* LPS are endotoxins. LPS consist of a hydrophobic lipid A region, a central core oligosaccharide region and a repeating O-linked polysaccharide portion[207]. Variations in the cell surface O-antigen is the basis for the 20 currently recognized serotypes of *P. aeruginosa*. LPS expressing many long O-side chains are termed smooth, whereas LPS expressing few, short or no O-side chain are termed rough. Two forms of the O-polysaccharide have been described: the A-band (homopolymer) and the B-band (heteropolymer)[207]. The O-antigenic region is highly immunogenic; however, whereas antibodies against the B-band LPS are highly protective in neutropenic mice, antibodies against the A-band LPS do not confer any protection[101]. When tested in animal models of acute infection, LPS-smooth isolates are significantly more virulent than LPS-rough isolates, clearly identifying the smooth LPS as an essential virulence factor[37, 193, 241]. The molecular mechanism of LPS-associated pathogenesis is linked to the induction of immunomodulatory molecules including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-8 and IL-10 by the lipid A region. The dysregulation of the host response induced by endotoxin leading to sepsis has been recently reviewed[29]. It has to be stressed that the *P. aeruginosa* lipid A is less toxic as compared to lipid A from enteric bacteria, a phenomenon possibly linked to the presence of mostly pentaacyl rather than hexaacyl chains[131]. *In vitro* studies have shown that whereas the LPS-smooth phenotype imparts serum resistance, rough mutants deficient in O-antigen synthesis are serum sensitive[39, 100]. This serum resistance is associated with B-band LPS[39]. In contrast to isolates recovered from the environment or from patients during acute infections, *P. aeruginosa* isolates recovered during chronic infections of CF patients are often rough and serum sensitive due to lack of B-band LPS[100, 133]. As the B-band O-antigens are highly immunogenic, the loss of these side

chains has been suggested to be part of adaptations during chronic infections that contribute to evasion of the host response[207]. Specific lipid A structures containing palmitate and aminoarabinose that are associated with resistance to cationic antimicrobial peptides and increased inflammatory response have been demonstrated in the LPS of CF isolates[57].

P. aeruginosa LPS is also involved in attachment to inert surfaces. Indeed modifications in production of either the A-band or the B-band LPS have been suggested to influence surface hydrophobicity and to affect adherence to polystyrene[148]. Moreover, changes of LPS phenotypes have been observed during biofilm formation and maturation. Such modifications were reversible and were lost when cells were removed from the biofilm and grown planktonically[13]. Cell-clumping that leads to microcolony formation during the initial step of biofilm formation might be due to physicochemical properties associated with the presence of certain LPS phenotypes[207].

Pier and colleagues have suggested that LPS might also act as a ligand for binding and entry into eukaryotic cells[273]. The outer core region of LPS has been suggested to be the ligand mediating binding to the CF transmembrane conductance regulator protein (CFTR) and ingestion in epithelial cells[189]. LPS modifications, therefore, could affect the efficient uptake of *P. aeruginosa* by host epithelial cells, a protective mechanism of the host that would facilitate bacterial killing by epithelial cell ingestion of *P. aeruginosa* followed by desquamation and swallowing. In contrast, during corneal infections, LPS has been proposed to be a ligand responsible for the invasion of the cornea, as LPS-induced uptake by corneal cells is not followed by destruction of the pathogen[146].

More recently, alteration of the LPS structure has been found to provoke important perturbations of the envelope organization leading to alterations of the type II secretion process of LasB elastase and lipase (LipA). It appears that LPS might be important for the stability of the type II secretion apparatus[158].

3. EXTRACELLULAR VIRULENCE FACTORS

Pseudomonas aeruginosa produces several extracellular products that, after the initial step of colonization, can cause extensive tissue damage, bloodstream invasion and dissemination (Figure 1). *In vivo* studies using mutants defective in the production of individual factors have shown which are essential for full virulence of *P. aeruginosa*. However, the relative contribution of a given factor may vary with the type of infection[167]. In the following section, the known biological effects of the most studied extracellular virulence factors associated with local tissue damage, dissemination and pathogenesis of acute *P. aeruginosa* infection, are summarized.

3.1. Virulence Factors Secreted via the Type I Secretion System

The type I (ABC-exporter) secretion pathway relies on three membrane proteins: the ATP binding cassette (ABC) protein AprD, the membrane fusion protein AprE and the outer membrane protein AprF[51]. So far, three proteins have been shown to be secreted via the type I secretion pathway in *P. aeruginosa*; alkaline protease, HasAp and AprX. HasAp is an iron-regulated heme-binding protein required for *P. aeruginosa* utilization of hemoglobin iron. Its role as a virulence factor is uncertain; however, it might play a role early during infection before inflammatory tissue damage has taken place and free iron becomes available[138]. The recently identified AprX protein is encoded within the *apr* determinant of the ABC- exporter system. Its function is unknown[51].

3.1.1. Alkaline Protease

Alkaline protease is a metalloprotease whose expression is controlled by the quorum-sensing circuit of *P. aeruginosa*[85]. The specific substrates of this enzyme are not clearly identified. Alkaline protease is produced *in vitro* by CF isolates[30, 145]. It has been suggested that alkaline protease might play a role in corneal infections. In one animal model of corneal infection, mutants deficient in alkaline protease production could not colonize traumatized corneas and did not produce the characteristic corneal damage observed with the parent strain[115]. Moreover, alkaline protease was detected in corneal tissue upon infection by *P. aeruginosa*[124]. However, these results have been recently challenged by studies using mouse and rat corneal infection models that were unable to demonstrate diminished virulence of alkaline protease deficient mutants[111]. Alkaline protease has been shown to degrade complement components, as well as interferon-γ (IFN-γ) and TNF-α[113, 178]. It also hydrolyzes fibrin and fibrinogen[225]. The role of alkaline protease in tissue invasion and systemic infections is unclear. An alkaline protease deficient strain was less virulent than its parent strain in a murine bacteremia model[110]. Interestingly, whereas immunization with either alkaline protease or ETA toxoid vaccines did not protect mice in a gut-derived *P. aeruginosa* sepsis model, combined alkaline protease and ETA toxoid vaccines improved survival from 7.1% to 60%[153].

3.2. Virulence Factors Secreted via the Type II Secretion System

The majority of extracellular virulence factors (LasA and LasB elastases, ETA, lipases, phospholipase C and protease IV) are secreted via the type II secretion pathway, or main terminal branch, of the general secretion

pathway (GSP), which occurs via a two-step process[65]. Exoproteins using this pathway are synthesized as precursors with an N-terminal signal peptide that is cleaved during transport across the cytoplasmic membrane by the Sec machinery. In the periplasm these proteins fold into their final conformation. The folded mature proteins are then translocated across the outer membrane via the Xcp secretion apparatus composed of 12 *xcp* gene products that form a complex machinery inside the bacterial cell envelope[65]. Interestingly, this secretion pathway has been suggested to be regulated by the quorum-sensing circuitry[23]. A second type II secretion system, named Hxc, responsible for the transport of the alkaline phosphatase LapA has been recently described in *P. aeruginosa*[8, 240]. Whether the Hxc secretion system is required for full virulence of *P. aeruginosa* remains to be investigated.

3.2.1. Exotoxin A

The ETA protein encoded by *toxA* is produced by most clinical *P. aeruginosa* strains[191]. ETA is a 68-kDa extracellular protein that enters eukaryotic cells by receptor mediated endocytosis. After cleavage in the endocytic vesicle, the toxin is translocated into the cytoplasm where it catalyzes ADP-ribosylation and inactivation of elongation factor 2, leading to inhibition of protein biosynthesis and cell death[259]. Furin, a member of the family of proprotein convertases, is involved in a separate two-step protein processing pathway that determines the sensitivity of target cells to ETA, the quantity of toxin receptor expressed on target cells and its proteolytic activation[214]. The characterization of the three-dimensional structure of ETA has allowed a better understanding of its mechanism of action and elucidation of the importance of each of its three domains[4]. Domain I comprises the N-terminus and is required for binding of ETA to target cells[116]. Domain II is required for translocation of ETA into the cytosol; deleting domain II reduces toxicity of ETA without abolishing cell binding[116]. Domain III possesses an endoplasmic reticulum retention sequence critical for intracellular targeting of the toxin and is responsible for the ADP-ribosylating activity of the toxin[24, 116, 224]. ETA is responsible for local tissue damage, bacterial invasion[265], and might have immunosuppressive activity[253]. Purified ETA is highly lethal for animals which supports its role as a major systemic virulence factor of *P. aeruginosa*[265]. ETA induces apoptosis of different eukaryotic cell types[191]. However, ETA induced-epithelial cell death involves an as yet unknown mechanism different from apoptosis, characterized by early mitochondrial dysfunction and superoxide anion production[191]. Evidence for *In vivo* production of ETA has been reported in CF patients. Indeed *toxA* transcripts accumulate in CF sputum[236], and ETA can be detected in sputum samples of CF patients[119]. Moreover, CF patients have high levels of circulating antibodies against ETA, and increased anti-ETA serum antibody levels strongly correlate with increased mortality[119, 162]. These observations suggest that the *in vivo*

production of ETA might be an important virulence determinant in CF patients. However, tissue damage in the lungs of CF patients does not consistently correlate with a high level of production of active ETA[82]. This could be due to mutations in *toxA* in CF isolates leading to ETA with reduced enzymatic activity and cytotoxicity[82]. In an acute animal infection model, it has been shown that ETA impairs host defenses during pneumonia by a mechanism independent of neutrophil influx and cytokine, chemokine or nitric oxide (NO) production[222]. In corneal infections, ETA mutants were rapidly cleared from the eye, suggesting that ETA might be important for the persistence and the ultimate infection of the eye[190].

3.2.2. LasB and LasA Elastases

The ability of *P. aeruginosa* to destroy elastin is considered to be a major virulence determinant during acute infection. The protein elastin accounts for a significant part of human lung tissue and is responsible for lung expansion and contraction capacities. Moreover, elastin is an important component of blood vessels which rely on it for their resilience. Elastolytic activity is believed to be responsible for the destruction of elastin containing human lung tissue, as well as for the pulmonary hemorrhages associated with invasive *P. aeruginosa* infections. The concerted activity of two enzymes, LasB elastase and LasA elastase, is responsible for elastolytic activity[83]. Both LasA and LasB elastases are zinc metalloproteases that act on a number of proteins including elastin. LasB elastase is a highly efficient protease, with a proteolytic activity about 10 times higher than *P. aeruginosa* alkaline protease and an activity toward casein about 4 times that of trypsin[83]. The LasA elastase is a serine protease that has staphylolytic activity[127], and that acts synergistically with LasB elastase to degrade elastin. LasA elastase nicks elastin, rendering it sensitive to degradation by other proteases such as LasB elastase, alkaline protease and neutrophil elastase[83].

Recently our understanding of the processing and transport of LasB and LasA elastase has improved. Both enzymes are synthesized as preproenzymes whose signal sequences are cleaved during the transport across the inner membrane[95, 125]. The propeptides act as intramolecular chaperones mediating the correct folding of the enzymes in the periplasm and the further processing by proteolytic cleavage[17, 154]. For LasB elastase, this cleavage is an autoproteolytic process that occurs in the periplasm and is catalyzed by the enzyme itself[154]. The propeptide of LasB elastase remains covalently bound to the mature enzyme and the complex only dissociates during or after the translocation across the outer membrane under the control of a host-specific factor[14]. In contrast, LasA elastase is transported in its unprocessed form, and either LasB elastase or a lysine-specific protease is required for the cleavage of the enzyme from its propeptide[126]. The propeptide is finally degraded by extracellular enzymes including LasB elastase[15]. The maturation of LasB elastase, required

for full proteolytic activity and stability, involves the formation of two disulfide bonds catalyzed by the thiol-disulfide oxidoreductase DsbA[16, 120].

Both LasB and LasA elastases have been found in the sputum of CF patients during pulmonary exacerbation[119] and transcription of the *lasA* and *lasB* genes has been demonstrated *in vivo* in the sputum of CF patients[236]. However, the relative role of LasB elastase in tissue destruction during the chronic phase of CF is less clear. It has been postulated that during this phase, antibodies present in high titers neutralize LasB elastase and that elastin damaged by minute amounts of LasA is degraded mostly by neutrophil elastase[83]. LasB elastase degrades not only elastin but also fibrin and collagen[107]. It can inactivate substances such as human immunoglobulins G and A[106], airway lysozyme[118], and complement components[113], as well as substances involved in the protection of the respiratory tract against proteases such as α-1-proteinase inhibitor[161] and bronchial mucus proteinase inhibitor[122]. Very intriguing is the recent observation that LasB elastase degrades the human surfactant proteins SP-A and SP-D[149]. These surfactant proteins are believed to play important roles in the innate immune response to *P. aeruginosa* and in the regulation of the inflammatory process by enhancing the removal of apoptotic neutrophils[149, 250]. Strikingly, reduced levels of SP-A and SP-D, as well as degradation products of SP-A, have been observed in bronchoalveolar fluid of CF patients colonized by *P. aeruginosa*[94, 149]. Degradation of SP-A and SP-D by LasB elastase could, therefore, be an important virulence mechanism during the chronic infection in CF patients not only by impairing the immune system, but also by increasing the inflammatory response[149, 250]. LasB elastase also impairs wound repair by altering cell motility and producing an imbalance between pro- and activated forms of matrix-metalloproteinase (MMP-2) and its inhibitor (TIMP-2)[43]. Therefore, LasB elastase not only destroys tissue components but also interferes with host defense mechanisms. Recently, LasB elastase has also been suggested to play a pathogenic role in chronic ulcers, characterized by excessive tissue proteolysis, by degrading kininogens, fibroblast proteins and proteoglycans, and inhibiting fibroblast cell growth[221]. LasB elastase also has immunomodulatory properties by activating the extracellular signal-regulated kinase (ERK) arm of mitogen-activated protein (MAP) kinases of alveolar epithelial cells leading to production of the potent pro-inflammatory cytokine IL-8[6].

The importance of LasA elastase during corneal infections is controversial. Whereas initial studies suggested that LasA mutants might have reduced virulence in a murine corneal infection model[194], this has been challenged in both mouse and rabbit corneal infection models in which LasA deficient mutants had similar virulence compared to the parent strains[2, 111]. In contrast, the importance of LasB elastase as a virulence factor has been clearly established by several studies in various animal infection models (burned mouse, acute and chronic rat pneumonia, mouse sepsis) in which mutants defective in

LasB elastase production were significantly less virulent than their parent strains[167, 239, 241].

3.2.3. Phospholipase C

P. aeruginosa produces a hemolytic and a non-hemolytic phospholipase C[176]. Whereas no pathogenic activity has been demonstrated for the non-hemolytic form, purified hemolytic phospholipase C (HPLC) has been shown to induce vascular permeability, organ damage and death in animal models[12]. Purified HPLC induces the release of inflammatory mediators from human platelets (12-hydroxyeicosatetraenoic acid), neutrophils (leukotriene B_4 and oxygen metabolites) and basophils (histamine), but reduces the production of IL-8 from monocytes[129]. Intravenous administration of HPLC induces significant tubular epithelial necrosis of the kidney with hematuria, while intraperitoneal administration provokes hepatonecrosis with cellular infiltration[157]. HPLC also suppresses neutrophil respiratory burst activity by interfering with a protein kinase C (PKC) specific signaling pathway[244]. It has, therefore, been suggested that HPLC might facilitate survival of *P. aeruginosa* in conditions characterized by an abundance of neutrophils such as bronchiectasis and CF[244]. The importance of HPLC as a virulence determinant has been demonstrated in animal infection models. Indeed, *P. aeruginosa* mutants deficient in HPLC production were less virulent than their parent strains in two mouse burn models of sepsis[177, 198], as well as in a chronic[91] and an acute[260] pneumonia model in rabbits.

3.2.4. Protease IV

Protease IV is a serine protease with a molecular mass of 26-kDa that specifically cleaves substrates on the carboxyl side of lysine-containing peptides[52]. This enzyme degrades the complement components C1q and C3, immunoglobulin G, fibrinogen, plasmin and plasminogen[52]. Recently, the catalytic site of protease IV has been identified and autodigestion found to be essential for the processing into a mature protease[245]. Protease IV is a significant virulence factor during corneal infections. *P. aeruginosa* mutants deficient in protease IV production are less virulent in rabbit and mouse corneal infection models[54, 168]; and purified protease IV restores corneal virulence of a protease IV deficient mutant[53]. Recently an endoprotease named PrpL, whose N-terminus is identical to protease IV has been characterized[261]. The production of this enzyme is regulated by the alternative sigma factor PvdS, itself regulated through the ferric uptake regulator (Fur)[261]. PrpL cleaves the iron-binding proteins lactoferrin and transferrin, as well as casein, elastin and decorin. Degradation of iron-binding proteins has been observed during chronic *P. aeruginosa* infections in CF patients[19]. It has therefore been postulated that PrpL might contribute to virulence in chronic *P. aeruginosa* lung infections[261].

3.2.5. Lipases

P. aeruginosa produces two lipases (LipA and LipC). As for other enzymes transported via the type II secretion apparatus, lipase maturation involves the formation of a disulfide bond catalyzed by DsbA[246]. Lipases might play a role in pathogenesis by degrading, in conjunction with HPLC, the major lung surfactant lipid dipalmitoylphosphatidylcholine[129, 151]. *P. aeruginosa* isolates from burn and CF patients frequently produce lipase, and lipase levels are elevated in bronchoalveolar lavage fluid from CF patients as compared to controls[129]. Purified lipase also enhances the induction of inflammatory mediators by HPLC[129]. It has therefore been postulated that the simultaneous production of lipase and HPLC might be deleterious to the host by inducing significant inflammation[129].

Other proteins secreted via the type II secretion pathway with so far no identified role during pathogenesis include an alkaline ceramidase[173, 174], an aminopeptidase[15] and a chitin-binding protein[71].

3.3. Virulence Factor Injection through the Type III Secretion Apparatus: The Exoenzyme S Regulon

In contrast to the above discussed exoproducts, which elicit pathological effects upon addition to eukaryotic cells and act distal from the site of infection, another type of exoproducts are not cytotoxic when added to eukaryotic cells. Instead, these exoproducts require close contact with target cells in order to elicit their toxicity at the site of infection. These exoproteins, including exoenzyme S, exoenzyme T, exotoxin U and exoenzyme Y, are secreted via the type III secretion pathway. This system uses a complex secretion and translocation machinery to inject, upon close contact, the effector proteins directly into the cytoplasm of target cells[9]. The genes encoding the secretion (*psc* and *pcr*), translocation (*popD and popB*) and regulatory (*exs*) machinery of this complex system are clustered together in a locus termed the exoenzyme S regulon on the *P. aeruginosa* chromosome[73, 76, 270]. The expression of the secretory, regulatory and structural loci of the type III secretion apparatus has been suggested to be coordinately controlled with the expression of effector genes (*exoS, exoT* and *exoU*) by a single transcriptional regulator ExsA in response to various environmental signals including low calcium and host cell contact[270]. However, a recent transcriptome analysis has revealed a hierarchical pattern of expression in which a subset of the type III secretion apparatus and regulatory genes are constitutively expressed regardless of the secretion-inducing signal or the presence of the positive regulator ExsA[264]. A membrane-associated adenylate cyclase termed CyaB was shown to control the expression of the type III secretion system via the cAMP binding protein Vfr[264]. This new signaling pathway

appears to coordinately regulate numerous host-directed virulence determinants including motility systems, attachment organelles and the type II secretory pathway, as well as the type III secretion apparatus[264].

The thiol-disulfide oxidoreductase periplasmic protein DsbA has also been recently shown to be required for the expression of the type III secretion system[96]. This enzyme catalyzes the formation of disulfide bonds required for the functional folding of proteins. It has been postulated that the DsbA protein might be required both for the expression of type III effector genes and the formation of the type III secretion apparatus[96]. Type III secreted cytotoxins are synthesized in the cytoplasm and often made competent for translocation via the type III secretion apparatus by a conformational change in a complex with a chaperone[9].

3.3.1. Exoenzyme S

Exoenzyme S (ExoS) is produced by about 40% of clinical isolates[232]. Both acute and chronic animal models of infection have suggested that whereas this exoproduct does not contribute to initial colonization, it might be responsible for tissue destruction[165] and for bacterial dissemination[164]. ExoS was initially purified as an aggregate composed of two proteins, a 49-kDa form that possesses ADP-ribosyltransferase activity termed ExoS, and a 53-kDa form with little intrinsic ADP-ribosyltransferase activity termed Exoenzyme T (ExoT)[9, 117, 166]. ExoS and ExoT share little amino acid sequence identity with other members of the family of bacterial ADP-ribosylating exotoxins. Surprisingly, ExoS aligns better with the vertebrate ADP-ribosyltransferases and shares some functional properties with them, including the ability to ADP-ribosylate multiple target proteins and to ADP-ribosylate more than one arginine residue within a target protein[9]. These observations suggest that ExoS might have an evolutionary link with the vertebrate ADP-ribosyltransferases. The ADP-ribosyltransferase activity of ExoS requires allosteric activation by the eukaryotic *F*actor *A*ctivating Exoenzyme *S* (FAS) protein[27, 128]. This protein belongs to the 14-3-3 eukaryotic protein family known to be involved in various functions including phospholipase A2 activity, regulation of tyrosine hydroxylase, tryptophan hydroxylase and possibly PKC activities[77]. ExoS is a bifunctional cytotoxin translocated into the cytoplasm of target cells during direct contact by the type III secretion system[75, 269]. Intracellular activity of the amino terminus of ExoS in eukaryotic cells disrupts actin filaments without cytotoxicity involving Rho-dependent GTPases[75, 185]. The carboxyl terminus of ExoS comprises an ADP-ribosyltransferase domain which is cytotoxic involving ribosylation of GTP-binding proteins such as Ras[28, 117, 254]. Both Ras and Rho contribute to tissue regeneration and wound healing[72, 213]. It has therefore been hypothesized that ExoS might contribute to chronic *P. aeruginosa* infections by inhibiting tissue regeneration and wound healing[9]. The ADP-ribosylating activity of ExoS has also been suggested to induce apoptosis of

host cells[123], and to eliminate cell adhesion and induce microvillus effacement[175]. ExoS might also contribute to pulmonary inflammation by inducing the synthesis of pro-inflammatory cytokines and chemokines[55], and by stimulating lymphocyte proliferation[10].

Besides its low ADP-ribosylating activity, ExoT is a GTPase-activating protein for the Rho signal transduction pathway[130]. It has therefore been suggested that ExoT might interfere with actin organization, exocytosis, cell cycle progression and phagocytosis[130].

3.3.2. Exotoxin U

Exotoxin U (ExoU), also termed PepA, is a third exoprotein secreted via the type III secretion system. The *exoU* gene shares with *exoS* and *exoT* similar promoter structures and an identical binding site for the transcriptional activator ExsA[66, 105]. Efficient secretion of ExoU requires a chaperone named SpcU[67]. Introduction of *exoU* into a collection of non-cytotoxic *P. aeruginosa* strains conferred a cytotoxic phenotype (associated with increased virulence in a murine model of acute pneumonia) to some but not all otherwise non-cytotoxic strains[3]. ExoU is expressed in most isolates from corneal infections[66]. In contrast, *exoU* is present in only 40% of strains isolated from acute respiratory tract infections in non-CF patients[105], and in only 10% of CF isolates[38]. The expression and secretion of ExoU by the type III secretion apparatus clearly result in cytotoxicity in mammalian cells. It has been postulated that the middle portion of the protein might allow the N- and C-terminal regions to functionally complement each other, and that ExoU toxicity might involve more than one activity[68]. Other studies in *Saccharomyces cerevisiae* showed that ExoU act by a similar mechanism in yeast and mammalian cells, but did not support the tethering model[197]. These authors suggested that ExoU might have phospholipase activity[197]. This enzymatic activity has been recently confirmed[215]. Recombinant ExoU was indeed found to have lipase activity *in vitro*, but only in the presence of yeast extract, suggesting that ExoU is a lipase that requires activation or modification by eukaryotic factors. The mechanism of ExoU cytotoxicity might therefore be explained by a broad substrate phospholipase activity destroying eukaryotic membranes, that contributes to necrotic cell death, cell permeability and rapid dissemination of ExoU-producing strains[215].

A type III secretion-dependent, ExoU-independent cytoxicity has been observed for *P. aeruginosa* laboratory mutants and several CF isolates suggesting the existence of a yet unidentified type III secreted toxin[26, 38, 103]. Apparently, macrophages and epithelial cells are differently affected by this type of cytotoxicity[26]. Whereas epithelial cells show significant morphologic modifications without decreased viability, macrophages are rapidly killed via the apoptotic pathway by *P. aeruginosa* laboratory strains[26]. Interestingly, CF isolates were reported to kill macrophages and neutrophils via a similar

type III secretion-dependent, ExoU-independent mechanism but in a process distinct from apoptosis termed oncosis[38]. Strikingly, this kind of cytotoxicity was observed for 6 out of 29 CF isolates[38]. Recently, the laboratory strain PAO1 was also shown to induce a type III secretion system-dependent apoptosis of mammalian epithelial cells[121]. This cytotoxicity was dependent on upregulation of CD95 on infected cells, as well as on bacterial adhesion, but not on internalization into the target cells[121]. Lung epithelial cell apoptosis mediated by the CD95/CD95 ligand system has been suggested to be an important host defense mechanism against *P. aeruginosa*[93]. Indeed upon respiratory challenge with *P. aeruginosa*, CD95- or CD95-ligand-deficient mice rapidly died from sepsis in contrast to normal mice which survived, leading to the hypothesis that activation of CD95 in lung epithelial cells may be essential for survival by increasing clearance of infected cells[93]. However, such a protective role for bronchial cell apoptosis has been contested by another study which suggested that airway epithelial cells are highly resistant to apoptosis[114]. Another mechanism of apoptosis induced by *P. aeruginosa* recently described involves ceramide release in sphingolipid-rich rafts[92]. Both CFTR, a suggested ligand for LPS[189] and CD95 are clustered in ceramide-enriched membrane signaling platforms after infection with *P. aeruginosa*. It appears that *P. aeruginosa* triggers ceramide release, leading to reorganization of these rafts, apoptosis and a massive inflammatory response due to release of IL-1[92]. Whether the previously described alkaline ceramidase[173, 174] is involved in this ceramide release remains to be investigated.

3.3.3. Exoenzyme Y

Exoenzyme Y (ExoY) is a fourth effector protein dependent on the ExoS regulon. This enzyme is an adenylate cyclase that is translocated into target cells via the type III secretion apparatus[271]. Apparently, a eukaryotic protein distinct from calmodulin stimulates the adenylate cyclase activity of ExoY. Intoxication of eukaryotic cells by ExoY leads to intracellular cAMP accumulation and a rounded morphology[271]. The role of ExoY in the pathogenesis of *P. aeruginosa* infections remains unclear.

3.3.4. Differential Expression of Type III Secreted Exoproducts: Invasion vs Cytotoxicity

Based on their ability to invade mammalian cells and to induce cytotoxicity, *P. aeruginosa* isolates can be classified into two distinct categories: (a) invasive strains that are not cytotoxic, (b) cytotoxic strains that are unable to invade epithelial cells[70]. Whereas both the *exoS* and *exoT* genes are found in invasive strains, cytotoxic strains seem to have lost *exoS* but retained *exoT*[69]. The cytotoxicity of non-invasive strains has been shown to result mainly from the action of ExoU[66, 105]. Cytotoxicity can be disabled by the inactivation of the

transcriptional regulator *exsA*, and such mutants are able to invade mammalian cells[59]. Moreover, defects in the PopB (PepB) and PopD (PepD) proteins, required for the translocation of effector molecules by the type III secretion apparatus, correlate with loss of cytotoxity and gain of invasion[104]. These observations have suggested the existence of an invasion-inhibitory activity that is regulated by ExsA in cytotoxic strains[59, 104]. Surprisingly, both ExoS and ExoT, known to be regulated by ExsA, inhibit invasion of cytotoxic strains but are secreted by invasive strains[36, 86]. This apparent contradiction can be explained by a differential expression of *exoS* and *exoT* during the exponential vs the stationary growth phases leading to a growth phase-dependent invasion phenotype[97]. ExoS and ExoT that are injected into host cells function as anti-internalization factors for both invasive and non-invasive strains. However, whereas invasive strains translocate much higher amounts of ExoS and ExoT into host cells during the exponential growth phase compared to the stationary growth phase, non-invasive strains translocate consistently high amounts of ExoT regardless of the growth phase[97]. It has been postulated that invasive strains might have evolved a mechanism to avoid intracellular killing and maintain their survival. In contrast, cytotoxic non-invasive strains apparently do not have such defenses, either because they have lost the capacity of intracellular survival or because they did not acquire it[97]. One attractive interpretation of these results is that invasive strains might adapt to environmental conditions; becoming invasive by turning off ExoS and ExoT secretion during stationary phase when nutrients are scarce and escaping inside host cells; and becoming cytotoxic during exponential growth when nutrients are abundant by turning on both ExoS and ExoT secretion, thus inhibiting internalization into host cells[97]. In contrast, cytotoxic strains seem to be unable to modulate their phenotype and kill host cells through the action of ExoU regardless of growth phase[97]. In this respect, invasive strains are more likely to induce chronic infections, whereas cytotoxic strains are more likely associated with acute localized disease involving tissue destruction. The importance of cytotoxicity for the pathogenesis of acute *P. aeruginosa* infections has been clearly demonstrated in a rabbit acute pneumonia model[132]. Whereas airspace instillation of the cytotoxic strain PA103 consistently caused alveolar tissue damage, progressive bacteremia and septic shock, instillation of an isogenic non-cytotoxic mutant, defective in the production of type III secreted toxins, did not cause a systemic inflammatory response despite a potent local inflammation in the lung[132]. Apparently, the bacterial cytotoxin-induced alveolar tissue damage is crucial for the pathogenesis of septic shock during *P. aeruginosa* pneumonia, as intravenous injection of the cytotoxic strain did not produce septic shock. This can be explained by a significant leakage of pro-inflammatory mediators (IL-8) into the bloodstream that stimulates TNF-α production and leads to the development of septic shock[132].

The prevalence of type III secretion genes has been recently analyzed in 115 clinical and environmental isolates from various origins[64]. All isolates harbored at least one of the type III secretion system structural genes and the *exoT* gene. In contrast, *exoY* (89% positive), *exoS* (72% positive) and *exoU* (28% positive) were variably present. An inverse correlation was observed between the presence of *exoU* and *exoS*. No difference was observed between clinical and environmental isolates. CF isolates harbored *exoS* more frequently than *exoU*, whereas urinary isolates showed a trend toward a reduced prevalence of *exoY*[64]. This study was limited as only genotypes were determined and no proof of secretion of functional exoproducts was available. Strikingly, in another collection of 29 CF isolates, even though 28 isolates possessed the type III secretion system, only 8 showed functional type III secretion of effector proteins[38].

Two recent clinical observations have suggested the importance of type III protein secretion as a major virulence determinant during *P. aeruginosa* infections[102, 208]. In the first study, the relative risk of death associated with lower respiratory tract and systemic infections with *P. aeruginosa* isolates expressing any type III protein was 6.2[208]. In particular, the structural type III secretion apparatus protein PcrV was associated with increased death[208]. A positive correlation was also observed between expression of type III secreted proteins in these clinical isolates and toxicity in a cellular model, as well as mortality in a murine infection model. Chronic colonization and subacute infections in CF patients were not associated with the type III secretory phenotype, confirming previous observations[38]. The authors concluded that detection of type III secretory proteins might help to distinguish respiratory tract colonization from potentially lethal infection[208].

One major criticism of this study is the lack of precise information concerning *P. aeruginosa* colonization vs real infection. *P. aeruginosa* frequently colonizes the respiratory tract of mechanically ventilated patients, and its isolation in sputum, tracheal aspirates and even bronchoalveolar lavage fluid from such patients is no proof of its pathogenicity. Such proof requires quantitative cultures associated with a compatible clinical picture. Unfortunately, no information about the definition of acute infection was provided. This significantly limits the value of this study and makes it difficult to draw any conclusions. Type III secretion is associated either with invasiveness or cytotoxicity. Strikingly, the authors did not discuss whether severe infections might be more associated with cytotoxicity as has been postulated previously[97, 132]. The questions raised by this first study have been recently answered by a well-designed retrospective study[102]. This study used precise definitions (quantitative bacteriology and suggestive clinical picture) to demonstrate the impact of *in vitro* expression of type III secreted proteins from *P. aeruginosa* isolates obtained by protected brush sampling or bronchoalveolar

lavage during 35 episodes of proven *P. aeruginosa* ventilator associated pneumonia. In all, 35 isolates, the presence of a type III secretion system gene (*popB*) confirmed that they harbored at least part of the ExoS regulon. Twenty-seven (77%) of the isolates produced at least one of the type III secreted proteins. Of these isolates, 22 (81%) had caused severe disease (defined as death or relapse despite adequate antimicrobial treatment). In contrast, only three out of eight (38%) type III negative isolates were cultured from patients with severe disease. This difference was statistically significant suggesting that *P. aeruginosa* isolates secreting type III proteins *in vitro* are associated with more severe disease. Ten isolates produced the cytotoxin ExoU. Nine (90%) of these ExoU-proficient isolates were isolated from patients with severe disease compared to three of eight (38%) ExoU-deficient isolates. This difference was also significant, suggesting that infections with ExoU-proficient isolates are associated with worse outcomes. In contrast, ExoS-proficiency was not associated with more severe disease.

Together, these two studies highly suggest that isolates producing type III secretion-dependent exoproducts, especially ExoU, are associated with severe disease in human infections. Therefore, patients infected with such strains might benefit from more aggressive therapies in order to prevent serious outcome. Moreover, passive immunization with antibodies raised against the type III translocation protein PcrV is protective in animal models[74, 218]. Therefore, strategies of passive immunization of patients colonized with type III secretion proficient *P. aeruginosa* isolates might be useful in order to prevent severe infections.

3.4. Other Extracellular Virulence Factors

3.4.1. Rhamnolipids

P. aeruginosa produces two hemolysins, phospholipase C (discussed previously) and rhamnolipids, that are proposed to act synergistically to break down lipids and lecithin. Both might contribute to tissue invasion by their cytotoxic effects. Rhamnolipids are rhamnose-containing glycolipid biosurfactants that have a detergent-like structure, and are believed to dissolve the phospholipids of lung surfactant, making them more accessible to cleavage by phospholipase C[142]. The resulting loss of lung surfactant may be responsible for the atelectasis associated with chronic and acute *P. aeruginosa* lung infection[143]. The genetic regulation of rhamnolipid production is complex and involves cell-density dependent control by the quorum-sensing circuit[147]. Rhamnolipids have been shown to inhibit the mucociliary transport and ciliary function of human respiratory epithelium[203]. Recently, rhamnolipids have been suggested to play an important role during the development of microbial communities inside biofilms[40, 58]. Indeed, an *rhlA* mutant unable to produce

rhamnolipids because of the inactivation of rhamnosyltransferase, one of the key enzymes in rhamnolipid synthesis, was incapable of maintaining open channels in the biofilm architecture. Moreover, it has been suggested that rhamnolipids may prevent colonization of *P. aeruginosa* biofilms by other planktonic microbes attempting to invade within the channels of its biofilm[40]. As the biofilm growth pattern is a major adhesion and colonization determinant, rhamnolipids could be considered an important virulence factor. Direct toxicity of rhamnolipids has been demonstrated in an amoeba (*Dictostelium discoideum*) host model[33]. However, the relative importance of rhamnolipids in acute or chronic infection has not been established.

3.4.2. Pyocyanin

Pyocyanin is a blue redox active phenazine pigment that generates reactive oxygen species due to intracellular redox cycling. Pyocyanin is reduced by NAD(P)H and the reduced pigment reacts with molecular oxygen to produce superoxide and hydrogen peroxidase exposing host cells to oxidative stress[20, 163]. It modifies host cells responses by inhibiting neutrophil superoxide generation and lymphocyte proliferation and by increasing IL-8 production by human airway epithelial cells[48, 163]. Pyocyanin has recently been shown to induce apoptosis of human neutrophils, an effect associated with rapid generation of reactive oxygen intermediates and lowering of intracellular cAMP levels[247]. It has also been postulated that pyocyanin could contribute to lung injury in CF by decreasing the ability of α-1 protease inhibitor to control the local activity of serine proteases[20].

3.4.3. Siderophores

Under iron limiting conditions, *P. aeruginosa* secretes two siderophores, pyoverdine and pyochelin, which compete with transferrin for iron. These iron-scavenging chemicals, chelate iron ions in the environment and the so-formed ferri-siderophores reenter the bacterial cells by means of specific cell-surface receptors such as FpvA[11, 134]. The iron is then released for incorporation into bacterial proteins. In addition to its role as an iron-scavenger, pyoverdine regulates the production of at least three virulence factors (ETA, PrpL endoprotease and pyoverdine itself)[134]. The production of ferri-siderophore receptor proteins is negatively regulated by the global iron regulator Fur (ferric uptake regulator)[251]. In animal models, pyoverdine and pyochelin are required for full expression of virulence of *P. aeruginosa*, apparently for both bacterial growth and dissemination[156, 238].

3.4.4. Hydrogen Cyanide

Hydrogen cyanide is a potent poison that blocks cytochrome oxidase leading to inhibition of mitochondrial respiration. This secondary metabolite

of *P. aeruginosa* is responsible for rapid paralytic killing of the nematode *Caenorhabditis elegans*[80]. Its role during human infections is unclear but it could participate in tissue destruction, and may contribute to toxicity in *P. aeruginosa* infected burn wounds[89].

3.4.5. Exopolysaccharides and Alginate

Bacterial biofilms consist of surface-attached organisms that live in highly differentiated communities inside an extracellular matrix composed of secreted polysaccharides, nucleic acids and proteins[34, 237]. The transition from free-swimming planktonic bacteria to sessile bacteria living in biofilms involves the initial attachment of the bacteria to the surface, followed by the accumulation of cell clusters and the formation of microcolonies, progressing ultimately to large macrocolonies separated by fluid-filled channels[34, 35, 78, 112]. Bacteria use growth inside a biofilm as a survival strategy because the surrounding matrix protects bacteria from phagocytes and complement activity[90, 112]. Biofilms also play an essential role in the colonization of indwelling devices such as catheters, endotracheal intubation devices and prosthetic implants. The ability to form biofilms has therefore been considered a virulence determinant in *P. aeruginosa* and other bacteria[112]. In CF patients, the most striking feature of persistent *P. aeruginosa* infections is the selection of mucoid mutants producing the exopolysaccharide alginate (a polymer of mannuronic and guluronic acid)[90]. It has long been assumed that alginate might also be the primary secreted polysaccharide in biofilms produced by environmental non-mucoid *P. aeruginosa* strains. However, recent studies involving two non-mucoid *P. aeruginosa* strains have indicated that alginate biosynthetic genes are not expressed and that alginate is not required during the formation of non-mucoid biofilms[266]. It has been hypothesized that oxygen radicals produced by neutrophils in CF patients might select strains harboring mutations in the *mucA* gene. This in turn could lead to the phenotypic change from non-alginate to alginate-producing strains[152]. The modified phenotype of *P. aeruginosa* growing in an alginate "slime matrix" has been shown to include resistance to antibiotics (e.g., aminoglycosides, β-lactam antibiotics, fluoroquinolones) and disinfectants[90]. The exact nature of the increased resistance to antibiotics of *P. aeruginosa* growing in alginate is unclear but has been attributed to several factors including slow growth, penetration barriers and β-lactamase production[112].

4. QUORUM SENSING

Due to its major role in the control of extracellular virulence factor production, the quorum-sensing circuit of *P. aeruginosa* could be considered a virulence determinant.

4.1. The Quorum-Sensing Circuit of *P. aeruginosa*

Several extracellular virulence factors, including LasB elastase and rhamnolipids are produced in a cell-density dependent manner under the control of the quorum-sensing (also termed cell-to-cell signaling) circuit composed of the *lasRI* and the *rhlRI* quorum-sensing systems[248]. The *lasRI* system is composed of *lasI*, the autoinducer synthase gene responsible for the synthesis of 3-oxo-C_{12}-HSL (*N*-(3-oxododecanoyl)-L-homoserine lactone, previously named PAI 1 or OdDHL), and *lasR* which encodes the transcriptional regulator LasR[84, 179, 181]. The *rhlRI* system is composed of *rhlI*, the C_4-HSL (*N*-butyrylhomoserine lactone, previously named PAI-2 or BHL) autoinducer synthase gene and *rhlR* which encodes the transcriptional activator RhlR[136, 171, 172, 182, 262]. At low cell density, the autoinducers 3-oxo-C_{12}-HSL and C_4-HSL are synthesized at basal levels and diffuse, or are transported[184], into the surrounding media where they become diluted; as a consequence, no gene transcription occurs. With increasing cell density, the two autoinducers accumulate until their intracellular concentration reaches a threshold level. At this critical concentration, they bind to their corresponding R-protein[79]. The R-protein/autoinducer complex is proposed to bind to specific DNA sequences upstream of target genes enhancing their transcription[234, 235]. These systems therefore allow the bacteria to communicate with each other (cell-to-cell signaling), to sense their own density (quorum sensing) and to behave in a coordinate manner, expressing specific genes as a population rather than individual cells[248].

4.2. Genes Controlled by the Quorum-Sensing Circuit

The *lasRI* system regulates *lasB* expression and is required for optimal production of other extracellular virulence factors such as LasA protease and ETA (Figure 2)[85]. This system has also been shown to induce the transcription of the *xcpP* and *xcpR* genes that encode proteins of the *P. aeruginosa* secretory pathway[23]. Therefore, quorum sensing controls virulence factor production directly at the level of expression and indirectly at the level of transmembranous export. The *rhlRI* system regulates the expression of the *rhlAB* operon that encodes a rhamnosyltransferase required for rhamnolipid production[170], and is also necessary for optimal production of LasB elastase, LasA protease, pyocyanin, cyanide, lipase and alkaline protease (Figure 2)[18, 136, 183, 205]. Therefore, like the *lasRI* quorum-sensing system, the *rhlRI* system regulates the expression of various extracellular virulence factors of *P. aeruginosa* (this has lead some authors to suggest another nomenclature, *vsm* for *v*irulence *s*econdary *m*etabolites). The *rhlRI* system has also been suggested to regulate the expression of *rpoS*, which encodes the stationary sigma factor σ^S involved in the regulation of various stress-response genes[135, 144]. Recently, these results

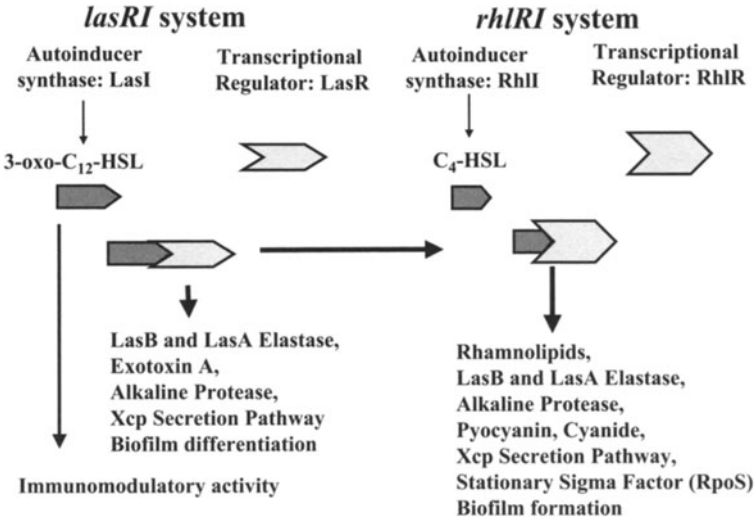

Figure 2. The quorum-sensing circuit in *P. aeruginosa*.

have however been challenged by other authors suggesting that instead *rhlI* transcription might be inhibited by RpoS[258].

Both quorum-sensing systems are highly specific in that the respective autoinducers are unable to activate the transcriptional activator protein of the other system[136, 183]. However, both quorum-sensing systems are not completely independent of each other (Figure 2). The LasR/3-oxo-C_{12}-HSL complex activates the expression of *rhlR* placing the *lasRI* system in a quorum-sensing hierarchy above the *rhlRI* system[135, 187].

Very recently, the general view of quorum-sensing control of *P. aeruginosa* gene expression has been significantly modified by transcriptome analysis. Preliminary studies using the screening of a library of random transposon (Tn5-*lacZ*) insertions in the genome of a *lasI/rhlI* mutant identified, in addition to the previously described virulence factors, 35 quorum-sensing controlled genes[257]. Using Affimetrix GeneChip genome arrays, two groups identified 315 vs 616 genes that are quorum-induced, and 38 vs 222 genes that are quorum-repressed[223, 255]. The important differences in both quorum-sensing induced and repressed genes between these two studies might be explained by different concentrations of exogenously added autoinducers, different growth media and growth conditions[252]. More importantly these studies showed that: (a) Quorum sensing controls not only virulence in *P. aeruginosa* but also many other genes involved in basic cellular processes, such as DNA replication, RNA transcription and translation, cell division and amino acid biosynthesis.

Moreover, quorum sensing controls genes involved in group behavior, such as chemotaxis and biofilm formation. (b) The timing of quorum-sensing activation is a continuum; some genes are activated during early growth whereas others are activated during exponential growth, or even during stationary phase. This timing is not dependent on the concentration of autoinducer, but on the availability of the transcriptional regulator proteins (LasR and RhlR). Indeed quorum-sensing promoters with high affinity for transcriptional regulators could titrate them during early growth, reducing their availability for other quorum-sensing controlled genes. As the level of transcriptional regulator proteins increases, additional quorum-sensing controlled promoters with lower affinity can be activated[223].

4.3. Quorum Sensing and Biofilm Formation

It has been shown that 3-oxo-C_{12}-HSL is required for the establishment of a differentiated *P. aeruginosa* biofilm[41], and that the autoinducer synthase gene *lasI* is expressed in a large number of cells during the initial phase of biofilm development[47]. In contrast, *rhlI* was expressed in fewer cells, and it has therefore been initially suggested that C_4-HSL might be of little importance during biofilm formation[47]. This was surprising as *P. aeruginosa* isolates from CF patients grown in biofilm produce, *ex vivo*, higher amounts of C_4-HSL as compared to 3-oxo-C_{12}-HSL[226]. This autoinducer production pattern has even been suggested as a marker for *P. aeruginosa* growing in biofilm in CF patients[226]. A similar elevated C_4-HSL/3-oxo-C_{12}-HSL ratio was found *in situ* in lung tissue of CF patients infected by *P. aeruginosa*[62]. Several other more recent studies have supported a role for the *rhlRI* quorum-sensing system during biofilm formation. This system is activated during the maturation stage of biofilm development[216], and might be important in CF patients for survival of *P. aeruginosa* growing in anaerobic conditions in biofilms[272]. Moreover, rhamnolipids, which depend on the *rhlRI* system, are required for the maintenance of biofilm architecture[40]. We have recently found that an isogenic *rhlI* mutant of the wild-type PAO1 strain PT5, unable to produce C_4-HSL, produced 70% less biofilm than its parent strain[61]. It appears that while the *lasRI* system is essential for biofilm differentiation[41], and plays a role during the irreversible attachment stage[216], the *rhlRI* system also plays an important role[61].

4.4. Quorum Sensing and the Immune Response

3-oxo-C_{12}-HSL alone has been suggested to contribute to the virulence of *P. aeruginosa* by altering the immune response to a *P. aeruginosa* infection in multiple manners[211]. This signaling molecule has been shown to inhibit

lymphocyte proliferation and the production of TNF-α by LPS-stimulated macrophages; affecting the Th1 vs Th2 balance in the host[243]. It stimulates human lung cells (fibroblasts, bronchial epithelial cells) to produce IL-8 (via NF-κB)[49, 228], stimulates T-cells to produce IFN-γ[230], and induces Cox-2 and PGE2 production in human lung fibroblasts[231]. Thereby 3-oxo-C_{12}-HSL might be an important modulator of host response during infection[229].

4.5. Regulation of the Quorum-Sensing Circuit

The regulation of the quorum-sensing circuit itself is very complex and involves several other transcriptional regulators. The *lasRI* system is regulated both positively and negatively at several levels. It has been shown to be positively controlled by the global response regulator GacA (which together with the sensor kinase GacS forms a two component system)[205], as well as by Vfr (a homologue of the global transcriptional regulator CRP) which is required for the transcription of *lasR*[1]. An inhibitor, called RsaL that represses the transcription of *lasI* has also been described[46]. Recently, QscR has been shown to inhibit the premature activation of *lasI* expression at low cell density[25]. The role of RpoS as a negative regulator of *rhlI* expression is still controversial[135, 258]. Moreover, the global post-transcriptional regulator RsmA modulates quorum sensing by inhibiting *lasI* and *rhlI* expression[188]. In addition, RsmA seems to affect virulence factor production downstream of the quorum-sensing circuitry, possibly at the level of structural genes[188]. The sigma factor RpoN has been suggested to exert a negative control on the quorum-sensing machinery. This effect is at least partially mediated by GacA[109]. Adding to this complexity is the finding of a *Pseudomonas* quinolone signal (PQS), that depends on the quorum-sensing circuit for its production, and which positively regulates *rhlI*, *rhlR* and *lasR* expression and induces *lasB* transcription[21, 81, 155, 186].

One important physiologic response to nutritional stress is the so-called stringent response, which primarily results in inhibition of stable RNA synthesis[22]. The effector of the stringent control is the nucleotide guanosine 3′,5′-bisdiphosphate (ppGpp), synthesized by the ribosome-associated RelA protein. Our laboratory has shown that the stringent response can activate the quorum-sensing circuitry in *P. aeruginosa* independently of cell density by changing the transcription pattern of regulatory genes and the kinetics of autoinducer production[249]. It has also been shown that both autoinducer and extracellular virulence factor production by *P. aeruginosa* are dependent on polyphosphate kinase (PPK), the enzyme responsible for the synthesis of polyP[202]. Given that polyP accumulation depends on multiple mechanisms including *relA* expression[5], it is possible that the mechanisms by which the stringent response influences quorum sensing is related to the regulatory

role of polyP[249]. In the environment as in the host, bacteria are likely to experience carbon or amino acid starvation independently of their own cell density. Hence *P. aeruginosa* could therefore be exposed to nutritional stress before a critical cell density has been reached. In this situation, the stringent response might prematurely activate the production of quorum-sensing regulated virulence factors. These factors would then provide the bacteria with new nutrients through their enzymatic activity, or allow them to disseminate to more favorable niches.

4.6. Quorum Sensing *in vivo*

The use of quorum sensing for the control of many of its extracellular virulence factors has been proposed to enable *P. aeruginosa* to overcome host defense mechanisms[248]. The production of extracellular virulence factors by a small number of bacteria would probably lead to an efficient host response neutralizing these compounds. However, the coordinate expression of virulence genes by an entire bacterial population, once a certain density has been reached, might allow *P. aeruginosa* to secrete extracellular factors only when they can be produced at high enough levels to overcome the host defenses. This could alter the precarious balance between host defenses and production of bacterial toxins, leading to invasion of blood vessels, dissemination, systemic inflammatory-response syndrome (SIRS) and finally death, as observed during acute overwhelming infections[248]. For these reasons, quorum sensing deserves to be considered as a virulence determinant.

Quorum sensing has been shown to be required for full virulence of *P. aeruginosa* in various animal infection models[211]. The *In vivo* production of the two autoinducers (AIs) has been demonstrated in a chronic pneumonia mouse model[267]. Animal infection models that have been used so far include acute pneumonia models in neonatal[180, 241] and in adult[230] mice, as well as an acute[137] and a chronic lung model[268] in rats and a burned mouse model[209, 212]. In these studies, *P. aeruginosa* strains mutated in *lasR*, *lasI* and *rhlI*, and therefore impaired in the production of quorum-sensing regulated virulence factors, induced significantly less tissue destruction, caused significantly less cases of pneumonia and disseminated disease, and were responsible for less mortality as compared to wild-type strains. Taken together, these studies demonstrate the importance of a functional quorum-sensing circuit for the virulence of *P. aeruginosa* during both acute and chronic infections. However, these quorum-sensing mutants were not completely avirulent, supporting the idea that the pathogenesis of *P. aeruginosa* infections is multifactorial, and that other, quorum-sensing independent factors also contribute to its virulence. The relative importance of quorum sensing during acute *P. aeruginosa* infection has recently been challenged by a study suggesting that quorum sensing

might inversely correlate with virulence in that 3-oxo-C_{12}-HSL might attract neutrophils *in vivo* and lead to more rapid clearing of the infection[217].

Only few data exist concerning the role of quorum sensing during *P. aeruginosa* infections in humans. Clinical isolates deficient in the production of extracellular virulence factors regulated by quorum sensing such as elastase and ETA have been described. Most isolates were specifically affected in the expression of the *lasB* and *toxA* genes[99, 196, 210], whereas quorum-sensing deficient clinical isolates have been described by only two reports[99, 233]. One *lasR* mutant still able to produce quorum-sensing signals has been isolated from a wound[99] and one *rhlR* mutant has been isolated from a patient with an urinary infection[233]. The production of AIs by clinical isolates has been demonstrated *in vitro* for *P. aeruginosa* strains isolated from CF patients[87], *ex vivo* by sputum of CF patients colonized by *P. aeruginosa*[226] and *in situ* in CF lung tissue[62]. These results have been recently confirmed by the detection of AIs in the sputum of CF patients[56, 159]. PQS has also been detected in respiratory samples of CF patients indicating that this other bacterial cell-to-cell signal is produced *in vivo* by *P. aeruginosa* infecting the lungs of CF patients[31].

In contrast, no data have been published so far concerning the production of AIs by *P. aeruginosa* strains colonizing mechanically ventilated patients. We have recently isolated AIs from biofilms covering endotracheal intubation devices retrieved from patients colonized by *P. aeruginosa*, demonstrating that AIs are effectively produced in this clinical setting (Favre-Bonté *et al.*, submitted). We also collected *P. aeruginosa* isolates colonizing the respiratory tract of intubated patients. Surprisingly, the *in vitro* production of virulence factors controlled by quorum sensing was significantly more frequent among isolates collected early, during the colonization, as compared to isolates collected later. We found three clinical isolates unable to produce quorum-sensing signals as a result of mutations in the *lasR* and/or *rhlR* transcriptional regulator genes (Dénervaud *et al.*, submitted).

As quorum sensing regulates the production of many extracellular virulence factors of *P. aeruginosa*, it has become an ideal target for new "anti-virulence" strategies. Such strategies include autoinducer antagonists[60, 108, 227], autoinducer degrading enzymes[50, 141, 206] and inhibitors of autoinducer synthases such as the macrolide azithromycin[61, 242].

5. OTHER RECENTLY DESCRIBED PUTATIVE VIRULENCE FACTORS

The enzyme adenylate kinase has been recently suggested to enhance macrophage cell death; therefore, it has been postulated to play a major

pathogenic role during the initial contact between *P. aeruginosa* cells and alveolar macrophages[150].

A *P. aeruginosa* serine/threonine protein kinase termed PpkA that is specifically induced within the host infection site, and required for full virulence in neutropenic mice, has been described[256].

A highly conserved genomic island termed PAGI-1 has been identified in about 85% of pathogenic isolates from various clinical sources[139]. This putative pathogenicity island might contain genes which allow *P. aeruginosa* to survive under stress conditions that generate oxygen radicals; however, its precise role in the pathogenesis of *P. aeruginosa* infections remains unclear[139].

6. CONCLUSIONS

As an opportunistic pathogen, *P. aeruginosa* is remarkably well equipped with many potential virulence factors that allow it (a) to adhere efficiently to epithelial cell linings as well as abiotic surfaces, (b) to compete with local flora and fight against local defenses in order to establish a persistent colonization and (c) to damage host tissues, disrupt defense mechanisms, invade deeper structures and elicit a strong inflammatory response. *P. aeruginosa* has developed impressive strategies to adapt to the human host, and to either become a chronic colonizer or an acute pathogen depending on the clinical setting of the host. With the availability of the entire genome sequence of *P. aeruginosa*, one can hope that a better understanding, on both the molecular and cellular level, of its interaction with host cells will lead to the development of more potent therapeutic and preventive strategies.

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2

ATP-UTILIZING ENZYMES, PURINERGIC RECEPTOR MODULATION, CUPREDOXINS AND MAMMALIAN CELL DEATH

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1. INTRODUCTION

Pseudomonas aeruginosa, like many other living aerobic organisms, uses oxidative metabolism for the generation of energy in the form of ATP. ATP (phosphoanhydride bond of ATP) hydrolysis liberates energy. ATP is also interconverted not only to other adenine nucleotides such as ADP, AMP or to adenosine, but allows phosphotransfer by an enzyme nucleoside diphosphate kinase (Ndk) to generate GTP, CTP, UTP, etc., or their deoxy derivatives, for RNA/DNA synthesis and participates in essentially all major metabolic reactions for carrying out cellular functions. Essentially all forms of living cells use ATP as a source of energy, and stores ATP or linear polymers of inorganic orthophosphate (polyphosphates) derived from ATP as sources of energy[33].

While ATP has intracellular roles as mediators of cellular energetics, it is now well established that ATP and other adenine nucleotides derived from it such as adenosine, are exported into the extracellular milieu where they carry out important biological functions[5, 28]. ATP is released by vesicular exocytosis from nerves, endothelial and epithelial cells[6, 32]. ATP binding cassette (ABC) proteins that carry out pumping of drugs out of the cells in an ATP-dependent

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manner are also believed to be involved in the transport of ATP across the outer membrane/plasma membrane, presumably through transporters or channels. An example of the ABC protein family is the cystic fibrosis transmembrane conductance regulator (CFTR) whose defect in human cells leads to the disease cystic fibrosis (CF). Patients suffering from CF, among other problems, accumulate abnormal fluids in their lungs that trigger bacterial infections, the most notable of which is that by *P. aeruginosa*[40, 47]. The involvement of CFTR with ATP transport was inferred based on the activation of outwardly rectifying chloride channels by extracellular ATP[56], but the role of CFTR in ATP release has since been disputed[60].

2. THE CONCEPT OF PURINERGIC RECEPTORS

As early as in 1929, Drury and Szent-Gyorgyi[21] reported the activity of several adenine nucleosides and nucleotides on heart and in 1959, Holton[29] demonstrated that during antidromic stimulation, the sensory nerves to the rabbit ear released sufficient ATP to produce significant changes in vascular tone, essentially defining a role of effluxed ATP in neurotransmission. The clear demonstration that ATP, or a related nucleotide, is a neurotransmitter in nonadrenergic, noncholinergic nerves supplying the gut or the urinary bladder was made by Burnstock *et al.*[7] that provided a sound basis for the role of extracellular ATP and other adenine nucleotides in neurotransmission, heart muscle activity and other biological functions. In immune cells, bacterial lipopolysaccharide dependent release of ATP has been reported in microglial cells and monocyte-derived human macrophages[22].

How does extracellular ATP or other adenine compounds derived from it act to modulate the numerous biological functions ascribed to them? Geoffrey Burnstock in 1972[8] coined the term 'purinergic' to put forward the purinergic neurotransmission hypothesis where the post junctional receptors for ATP were postulated to account for ATP action, even considering the high variability in the activities of adenine nucleoside and nucleotides in a wide variety of tissues[9]. To account for such variability, Burnstock[10] subsequently suggested that the purinoreceptors comprised of two classes: Class P1 receptors responsive to adenosine and class P2 receptors activated by ATP and ADP. This classification was, however, complicated by the fact that membrane-bound ectoenzymes could convert extracellular ATP to AMP or adenosine whereby ATP could be seen to activate both P1 and P2 types of receptors[43]. P2 receptors were further divided into several classes, P2X purinoreceptors with ATP as an agonist, P2Y purinoreceptors with 2 methylthio ATP as an agonist[11], P2T purinoreceptor activated by ADP and P2Z purinoreceptor activated by an ionic form of ATP, ATP^{4-}, and present in

immune cells such as macrophages, mast cells and lymphocytes[24]. Currently, P1 receptors have been classified to four G-protein coupled receptor classes A_1, A_{2A}, A_{2B} and A_3, while P2 receptors have been divided into G-protein coupled P2Y receptors and ligand-gated ion channel P2X receptors. Cloning of seven P2X receptors, termed $P2X_1$ to $P2X_7$, and eight P2Y receptors, termed $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$, have been accomplished[1, 12, 20, 52].

The presence of purinoreceptors that are activated by various adenine (and uracil) nucleotides and that are present on the surface of various mammalian cells suggests that such cells have evolved intricate mechanisms that control their functionality in response to inside or outside signals. Indeed, interconversion of various nucleotides through membrane-bound nucleotide-interconverting enzymes such as ecto-nucleotidases, ecto-nucleoside diphosphate kinase and others[36, 67] may produce a pool of nucleotides that act as agonists of various P1 and P2 purinoreceptors, greatly modulating their functionality. While the exact roles of various mammalian ecto-nucleotide interconverting enzymes in individually modulating specific purinergic receptors have not been evaluated as yet, it is clear that such enzymes play an important role in the generation of nucleotides that in turn modulate many cellular functions including immune cell functions such as cytokine release, platelet aggregation, mast cell degranulation and various inflammatory responses[20, 31, 37, 46].

2.1. Purinoreceptors on Immune Cells: P2X and P2Y Family Receptors

P2X receptors are characterized by two transmembrane domains, short intracellular N- and C-termini and an extracellular loop with 10 conserved cysteine residues. These receptors are ATP activated ion channels. One variant, originally called P2Z but subsequently shown to belong to a new class $P2X_7$[59], is found in both nonimmune and immune cells[17] but are particularly abundant in macrophages and mast cells and in microglia of the central nervous system (CNS). The microglia normally provide a defensive inflammatory response to infections and tissue damage in CNS[34], very similar to a role macrophages play in the immune system[19]. The $P2X_7$ receptor has been implicated in the lysis of antigen-presenting cells by cytotoxic T lymphocytes, in the mitogenic stimulation of human T lymphocytes, formation of multinucleated giant cells as well as in the opening of transmembrane pores which are permeable to hydrophilic molecules of molecular weight up to 900 Da. Such transmembrane pore opening in macrophages by ATP through $P2X_7$ receptor activation allows macrophage cell death with consequent death of any engulfed pathogen[35]. $P2X_7$ receptor activation has also been shown to induce activation of multiple

caspases known to be involved in apoptotic cell death in myeloic cells[23]. The activation of cell surface P2Z ($P2X_7$) receptors for induction of cell death through efflux of intracellular ATP to the outside is a strategy that a microphage uses when it finds itself unable to process an intracellular pathogen such as *Mycobacterium bovis*[35]. In contrast to P2X receptors, P2Y receptors which are ATP-activated G-protein-coupled receptors have seven transmembrane domains with an extracellular N- and an intracellular C-terminus[13]. Both ATP and UTP can act as agonists for activation of certain P2Y receptors such as $P2Y_2$ or $P2Y_4$. In macrophages, P2Y receptors are involved in IL-1β maturation and release, modulation of phagocytosis, superoxide generation and enhancement of nitric oxide (NO) synthase and lipopolysaccharide expression[13]. ATP or UTP analogs are known to induce chloride secretion by CF epithelial cells and thus demonstrate to have a positive effect on mucociliary clearance. ATP and UTP are known to stimulate CF epithelial cells through P2Y receptors. It is thus interesting to note that *P. aeruginosa*, which causes chronic infections in the lungs of CF patients[40, 47], secretes quorum-sensing signaling molecules that inhibit expression of P2Y receptors in CF tracheal gland cells, thereby exacerbating the severity of the infection[54].

2.2. Secretion of ATP-Utilizing Enzymes by *P. aeruginosa* and Other Pathogens

As mentioned earlier, the mammalian cell surface-bound ecto-enzymes greatly modulate the nature of adenine nucleotide pools. The presence of ecto-nucleoside diphosphokinase (Ndk) allows transfer of terminal phosphate from ATP to UDP producing UTP, which is an agonist for various P2Y receptors[13]. It is thus conceivable that various pathogenic bacteria might secrete adenine nucleotide interconverting enzymes during host cell infection to modulate host cell purinergic receptors to their own advantage. The secretion of enzymes such as ATPase and Ndk to the growth medium by *M. bovis* BCG was reported in 1999 by Zaborina *et al.*[64]. They demonstrated that the secretion of these two ATP-utilizing enzymes depended upon the presence of eukaryotic proteins in the growth medium which seemed to facilitate the opening of the secretory apparatus. Filtered growth medium containing these enzymes prevented ATP-induced macrophage cell death, suggesting that these enzymes were released to prevent macrophages from undergoing ATP-inducible P2Z ($P2X_7$) receptor mediated cell death which allows death of the *M. bovis* cells, as reported by Lammas *et al.*[35]. As an intracellular pathogen growing within macrophages, it is important for *M. bovis* to prevent macrophage cell death early on to allow its own replication.

While *M. bovis* is an intracellular pathogen growing within macrophages, *P. aeruginosa* is an extracellular pathogen that colonizes and forms a biofilm in the lungs of CF patients[58]. As an extracellular pathogen, *P. aeruginosa* has no particular need to prevent macrophage cell death. Since the CF patients are immunocompetent, it is more important for *P. aeruginosa* to prevent phagocytosis and kill the macrophages. Indeed, Zaborina *et al.*[63] reported that a mucoid CF-isolate strain of *P. aeruginosa* secreted multiple enzymes with Ndk, ATPase, adenylate kinase (Ak), 5′-nucleotidase and other enzymatic activities. The growth medium containing these enzymes had substantial cytotoxicity toward macrophages which was abolished in oxidized ATP (oATP)-pretreated macrophages, suggesting a major role of P2Z receptor activation in such cytotoxicity. Similar to the secretion of ATPase and Ndk by *M. bovis* BCG, the secretion of *P. aeruginosa* ATP-utilizing enzymes was enhanced in presence of eukaryotic proteins such as kappa casein. Zaborina *et al.*[63] further demonstrated the formation of AMP and adenosine, which act through activation of P1 receptors, from ATP, suggesting that multiple receptor activation due to generation of a pool of adenine compounds contributes to enhanced macrophage death. Indeed, purification of a single enzyme, Ak, from the growth medium of *P. aeruginosa* and its effect on macrophages in the presence of its substrates demonstrated high cytotoxicity, indicating its role as a potential virulence factor[39]. A similar role for 5′-nucleotidase is likely since bacterial 5′-nucleotidase purified from *Haemophilus influenzae*[66], as opposed to mammalian 5′-nucleotidase which uses AMP as a substrate, can use various nucleotides such as ATP or ADP, thereby producing adenosine from ATP, as is seen when *P. aeruginosa* filtered growth medium is incubated with ATP[63]. As mentioned previously, adenosine and AMP activate additional receptors belonging to P1 class, thus contributing to enhanced macrophage cell death. A schematic diagram showing these steps is presented in Figure 1.

It should be emphasized at this point that *M. bovis* BCG or *P. aeruginosa* are not the only pathogens that secrete ATP-utilizing enzymes to the outside medium[63, 64]. Other bacterial pathogens such as *Burkholderia cepacia*[41] and *Vibrio cholerae*[49] have been shown to secrete such enzymes in their growth medium which contribute to macrophage and mast cell death. More recently, the parasitic nematode *Trichinella spiralis*, which causes major human intestinal inflammation and damage, has been reported to secrete such exoenzymes as 5′-nucleotidase, Ndk, apyrase and adenosine deaminase[26, 27]. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, has also been reported to secrete Ndk and indeed purified Ndk was shown to act as a cytotoxic factor for macrophages, helping in the bacterial dissemination and evasion of the immune system[15].

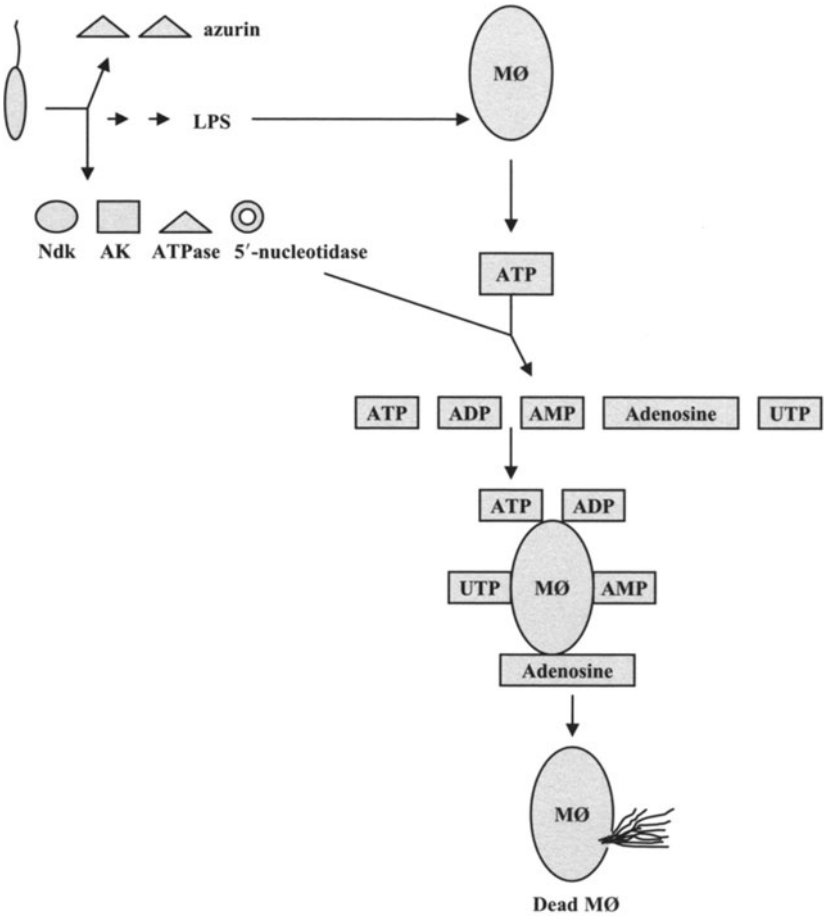

Figure 1. A schematic diagram of the events associated with the release of enzymes normally involved in cellular energetics in *P. aeruginosa* but which show profound cytotoxicity toward macrophages (MØ). Two types of enzymes released, cupredoxin such as azurin and ATP-utilizing enzymes such as Ndk, AK, etc., are shown. The release of cell wall lipopolysaccharide (LPS), presumably because of some lytic events, allows macrophages to efflux ATP. The ATP is then acted on by the ATP-utilizing enzymes released from *P. aeruginosa* to generate various adenine and uracil nucleoside/nucleotides through phosphotransfer by Ndk. These compounds, in turn, bind to the purinergic receptors on macrophage cell surface, thus contributing to their cell death through surface pore formation and leakage of low-molecular weight compounds; some purinergic receptor activation may also occur and lead to macrophage apoptosis.

3. SECRETION OF CYTOTOXIC FACTORS BY *P. AERUGINOSA* AND *B. CEPACIA* THAT OPERATE IN THE ABSENCE OF ATP-INDUCIBLE PURINERGIC RECEPTOR MODULATION

During column chromatographic purification of various ATP-utilizing enzymes from the growth medium of *P. aeruginosa*, Zaborina *et al.*[63] obtained a fraction that had ATP-independent high cytotoxicity toward macrophages. This fraction allowed macrophage killing without requiring activation of the P2Z receptors. Zaborina *et al.*[65] subsequently demonstrated that after column chromatographic fractionation of the growth medium of *P. aeruginosa* strain 808, a Q-Sepharose column flow through (QSFT) fraction enriched with two redox proteins azurin and cytochrome c_{551} demonstrated high cytotoxicity toward macrophages. This cytotoxicity was due to induction of apoptosis as evidenced by the activation of procaspase-3 to caspase-3. Using purified azurin and cytochrome c_{551}, Zaborina *et al.*[65] confirmed the role of these redox proteins in entering macrophages and triggering caspase-mediated apoptosis in such cells. Azurin was shown to be more efficient in killing macrophages than cytochrome c_{551}[65].

3.1. Azurin is a Member of the Cupredoxin Family of Redox Proteins

Azurin is a member of a group of proteins collectively called cupredoxins because they are small, soluble redox proteins whose active sites contain a type I copper[18, 44]. All of them participate in a wide range of electron transport reactions with various electron transfer partners such as cytochromes and nitrite reductases (NIR) with haem or Cu as their prosthetic groups participating in anaerobic respiratory denitrification processes or photosynthesis or various dehydrogenation reactions. The Cu NIRs have been divided into two subgroups, blue or green depending on their color, which are believed to have two specific redox partners, azurins and pseudoazurins. Both azurins and pseudoazurins are involved in denitrification by different bacteria. *Alcaligenes xylosoxidans* produces two azurins, azurin I and II, which have a sequence identity of 68%. In contrast, *P. aeruginosa* produces a single type of azurin. Some bacteria such as *Alcaligenes faecalis* S-6 or *Achromobacter cycloclastes*[55] produce pseudoazurin which has low sequence identity with azurin (less than 20%) and acts as the electron donor to a NIR cytochrome oxidase or cytochrome cd1. The protein contains eight β-strands which form two β-sheets giving an overall β-sandwich topology[55]. Another important group

of Cu proteins is the plastocyanins found in cyanobacteria, algae and plants. The approximately 11 kDa plastocyanins have an overall structure of an anti-parallel β-barrel that is conserved among cyanobacteria and higher plants. The plastocyanins play a role in the photosynthetic process by shuttling electrons from cytochrome f to photosystem I[57] in cyanobacteria, algae and plants. Interestingly, the electrostatic and hydrophobic features of the cyanobacterial plastocyanins are much more akin to those of azurins than to those of plant plastocyanins, providing interesting clues to a possible common mode of action.

In addition to azurin, pseudoazurin and plastocyanin, there are less studied cupredoxins such as rusticyanins, amicyanins and stellacyanins[18]. Rusticyanin is involved in the iron respiratory chain of the bacterium *Thiobacillus ferrooxidans* where it is believed to shuttle electrons from cytochrome c_{552} to cytochrome oxidase. Stellacyanins are plant-derived Cu proteins that are believed to be involved in redox reactions with small compounds rather than carrying out electron transfer reactions. Amicyanins are involved in the transfer of electrons from methylamine dehydrogenase to a c-type cytochrome in the methylamine-oxidizing electron transport pathway of methylotrophic bacteria such as *Paracoccus denitrificans*. While the overall structural fold of amicyanin is very similar to that of plastocyanin, the main difference being a large extension in the N-terminus of amicyanin, the electrostatic potential distributions in amicyanin are highly dipolar and similar to those of pseudoazurins[18]. Indeed, *Methylobacterium extorquens*, when grown in the presence of methylamine and copper, produces primarily a pseudoazurin rather than an amicyanin.

X-ray crystallographic studies, kinetics, gene sequence analysis and molecular electrostatic potential determinations have provided a wealth of data on the structure–function relationships of various cupredoxins. A hydrophobic patch on the protein surface adjacent to the copper site is a common feature of this family of proteins and is believed to be involved in protein–protein interactions with their partners during electron transfer, for example between *P. aeruginosa* azurin and its partners cytochrome c_{551} and nitrite reductase. There is often considerable genetic sequence identity among cupredoxins isolated from different sources. For example, the sequence identity among various azurins isolated from different bacteria ranges from 60% to 90%. In contrast, pseudoazurin, which is also involved in denitrification, exhibits low sequence identity ($<$20%) with azurin but has high structural identity. There are two basic differences between the overall structures of azurins and pseudoazurins. There is a carboxy terminus extension in pseudoazurin, as compared to azurin, that has two α-helices. Second, in its middle part, azurins contain an extended loop that is considerably shortened in pseudoazurin. The most notable difference is, however, in the hydrophobic patches of these two

cupredoxins. There are four surface Lys residues which form part of a lysine ring around the Cu site in pseudoazurin. Mutations in such a lysine ring resulted in significant reductions in the affinity of pseudoazurin with its partner nitrite reductase (NIR) from *A. faecalis* S-6, signifying possible protein–protein interaction between the site containing the lysine ring with the negatively charged surface residues near the type I Cu site of NIR. Unlike pseudoazurin, azurins do not have a lysine ring around their hydrophobic region, although azurins from *Alcaligenes denitrificans* or *Alcaligenes xylosoxidans* have two Lys residues near the hydrophobic patch while azurin from *P. aeruginosa* has a single Lys residue in this area. Thus, while the hydrophobic patch near the type I Cu site in azurins is essentially neutral, pseudoazurin has a positive potential at the hydrophobic patch, but also a localized negative charge opposite the hydrophobic patch, giving pseudoazurin a dipole. Such differences in the charge content of the hydrophobic patches of azurin and pseudoazurin may explain the fact that in contrast to azurins that react very sluggishly as electron donors to the green nitrite reductase, pseudoazurin can readily donate electrons to both blue and green copper nitrite reductase[44]. Whether pseudoazurin exhibits cytotoxicity to mammalian cells similar to azurin remains to be seen.

3.2. Azurin Demonstrates Cytotoxicity Toward J774 Cell Line Derived Macrophages

While a great deal is known about the structure–function relationships and electron transfer activities of various cupredoxins, very little is known about their cytotoxic effects on macrophages or any other biological properties. The ability of azurin and cytochrome c_{551} to induce apoptosis in macrophages[65] therefore raised an important question: Is azurin by itself cytotoxic? Yamada *et al.*[61] clearly demonstrated that a purified preparation of azurin had high cytotoxicity toward J774 cell line derived macrophages which are a murine reticulum cell sarcoma whose ascites form has the macrophage properties[53]. TUNEL assay, caspase assays and flow cytometric analysis clearly demonstrated that azurin induced apoptosis in macrophages. When purified *P. aeruginosa* azurin was incubated with J774 macrophages, azurin entered the cells and was localized in the cytosol and nuclear fractions of the macrophages but not in the mitochondria. Correspondingly, azurin elicited an enhancement in the intracellular level of the tumor suppressor protein p53. Subcellular fractionation studies demonstrated increasing levels of p53 in the cytosol and nuclear fractions during incubation of the macrophages for 0, 3, 6 and 12 hr with azurin. Enhanced levels of p53, which is a known transcriptional activator of the apoptogenic protein Bax and a known inducer

of apoptosis, allowed increased Bax trafficking to the mitochondria, resulting in the release of mitochondrial cytochrome c into the cytosol. The release of mitochondrial cytochrome c into the cytosol is an important event in apoptosis[2] and treatment of the macrophages with azurin clearly triggered this process. Some mammalian redox proteins enhance intracellular p53 levels through complex formation with p53. To determine how treatment with azurin leads to elevated intracellular level of p53, Yamada *et al.*[61] measured the ability of wild type (wt) azurin to form complexes with p53 using glycerol gradient centrifugation analysis. They also used two site-directed mutant (M44KM64E and H46G) azurins, which demonstrated low levels of cytotoxicity toward macrophages, for their ability to form complexes with p53. In case of wt azurin, oligomeric p53-azurin complexes were detected in the glycerol gradients while the mutant azurins were deficient in forming such oligomeric complexes with p53. Yamada *et al.*[61] further demonstrated that complex formation with azurin allowed stabilization of p53. p53 is normally a labile protein with a half life of a few minutes. The azurin–p53 complex was more stable with a higher half life. Since p53 is normally susceptible to proteolytic cleavage because of its complex formation with another protein Mdm2 at the N-terminal, which promotes ubiquitination of p53 leading to its degradation[30], it is likely that wt azurin competes with Mdm2 by forming a complex at the N-terminus of p53, thereby preventing p53 ubiquitination and proteolysis (Figure 2).

Many p53 complexes with mammalian redox proteins are known to generate reactive oxygen species (ROS), which are potent inducers of apoptosis. To determine if azurin–p53 complexes can give rise to higher levels of ROS or if antioxidants could remove such ROS, thereby reducing the level of cytotoxicity, Yamada *et al.*[61] measured ROS levels in live macrophage cells either not treated or treated with azurin. Treatment with wt azurin produced higher ROS levels as compared to untreated controls. Most importantly, when macrophages were pretreated with antioxidants such as superoxide dismutase (SOD) or Trolox, a vitamin E analog, before treatment with azurin, the cytotoxicity levels were greatly lowered in antioxidant-pretreated macrophages, clearly suggesting that higher levels of ROS generation within macrophages during azurin treatment allowed high level of cytotoxicity through induction of apoptosis.

Is secretion of azurin by *P. aeruginosa* the only example of a cupredoxin being elaborated by a pathogen for killing of macrophages? Punj *et al.*[51] have recently reported that azurin is also secreted by strains of *B. cepacia* isolated from the lungs of CF patients and belonging to different genomovars. A purified azurin homologue from a *B. cepacia* strain was shown to induce apoptosis in macrophages in a caspase-dependent manner and was localized in both the cytosol and nucleus when incubated with or microinjected into macrophages.

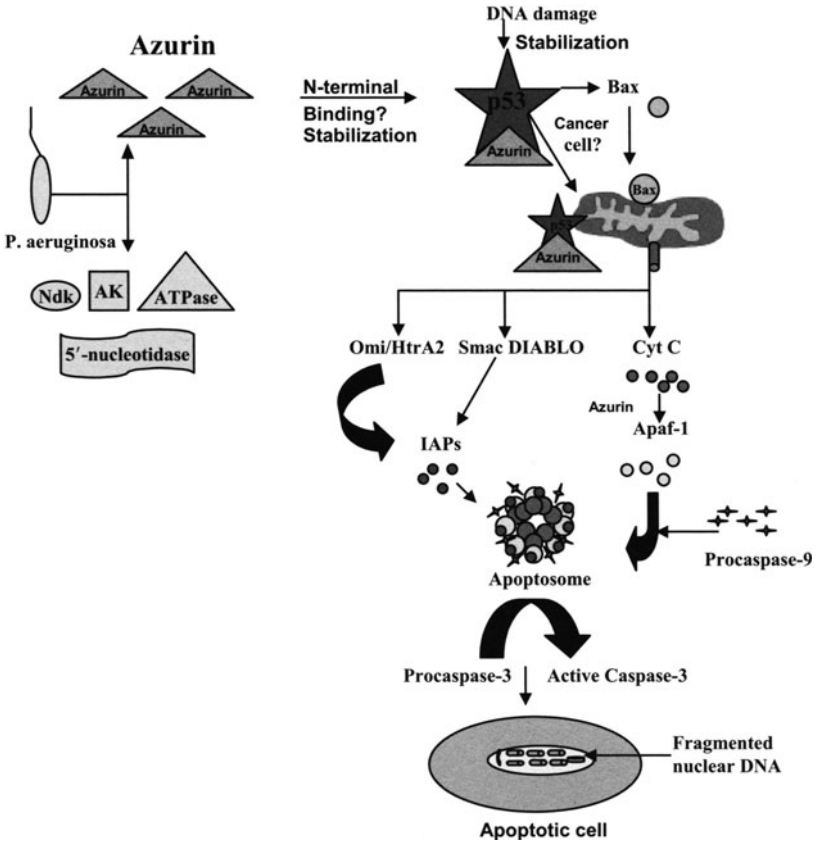

Figure 2. Release of a cupredoxin azurin from *P. aeruginosa* and its mode of action. As shown in Figure 1, in addition to the ATP-utilizing enzymes, a cupredoxin such as azurin is released from *P. aeruginosa*, particularly in response to the presence of eukaryotic proteins. The released azurin then enters macrophage cells and binds to the tumor suppressor protein p53. Such binding and complex formation contribute to stabilization of p53[61]. The binding of azurin in the N-terminus of p53 is believed to interfere with the binding of Mdm2 and is speculative. The stabilization of p53 leads to higher levels of intracellular p53 which then enhances the level of the proapoptotic protein Bax or in some cancer cells can directly traffic to the mitochondria. This allows the release of mitochondrial cytochrome c to the cytosol because of a loss of mitochondrial membrane permeability. In the macrophage cytosol, the cytochrome c forms a multimeric complex with Apaf-1 and Procaspase-9 (apoptosome), leading to the formation of active caspase-9 which in turn activates the caspase cascade. Activation of the caspase cascade triggers apoptosis[2].

Since azurins are produced by a variety of bacteria, both pathogenic and non-pathogenic[18, 25, 44, 50], it would be of interest to examine whether all forms of azurins retain this cytotoxic property or whether azurin might be secreted specifically by pathogenic bacteria.

3.3. Redox Activity of Azurin is not Important for its Cytotoxicity

An intriguing role of azurin in the induction of apoptosis in macrophages is a lack of involvement of the redox activity in this process. Goto *et al.*[25] isolated apo-azurin devoid of copper and several site-directed mutant azurins such as Cys112Asp (C112D) and Met44LysMet64Glu (M44KM64E), based on the fact that the cysteine residue (Cys112) is involved in coordination with copper and therefore is an important residue for electron transfer. The two Met residues (Met44 and Met64) are near the Cu site in a hydrophobic patch that is believed to be involved in the interaction of azurin with its electron transfer partners such as cytochrome c_{551} or nitrite reductase. Indeed, mutational replacements of these residues greatly reduced the efficiency of electron transfer between reduced cytochrome c_{551} and oxidized azurin[25]. When tested for complex-forming ability with the tumor suppressor protein p53, wt azurin and C112D mutant azurin demonstrated a high level of complex formation with oligomeric p53 while the M44KM64E mutant azurin seemed to be deficient in producing oligomeric p53 complexes. Interestingly, the wt azurin, C112D mutant azurin and apo-azurin, which were proficient in complex formation with p53, demonstrated high cytotoxicity and produced higher level of ROS, while incubation of macrophages with M44KM64E mutant azurin, defective in oligomeric complex formation with p53, had low cytotoxicity and was much less efficient in generating ROS. Cytotoxicity induced by wt, apo- or C112D mutant azurin was completely abolished in the presence of the antioxidant Trolox, thus demonstrating the role of ROS as the mediator of cytotoxicity. Pretreatment of macrophages with catalase had no effect, whereas pretreatment of macrophages with SOD greatly reduced azurin-mediated cytotoxicity, thus demonstrating a key role for superoxides in the induction of apoptosis[25].

If redox activity of azurin is not important for its cytotoxicity, then how does azurin trigger apoptosis in mammalian cells? It has recently been demonstrated that the redox activity of other apoptosis-inducing mammalian redox proteins is also not needed for their apoptogenic functions[42, 45]. Redox proteins such as cytochrome c and apoptosis inducing factor (AIF), released from the intermembrane space of mitochondria to the cytosol, are known to be important in the triggering of apoptosis[2]. It is, however, not clear if the redox activity of mitochondrial cytochrome c is important for its apoptogenic activity. Mitochondrial cytochrome c is known to form an apoptosome through multimerization and complex formation with a cytosolic protein Apaf-1 which also recruits procaspase-9. The cleavage of procaspase-9 to active caspase-9 then leads to the activation of caspase-3 and other members of the caspase cascade. We have recently hypothesized[50] that the presence of redox proteins, that are

normally localized in the mitochondria, in the cytosol sends a message to the eukaryotic cell that the mitochondria are damaged. The mitochondria in the eukaryotic cells are believed to have bacterial ancestry. Over 1.7 billion years ago, when the earth was moving from an anaerobic environment to increasingly oxygen-rich environment, the bacteria being highly adaptable, developed the capability to use molecular oxygen for their energy and survival. The eukaryotic cells being less adaptable took the route of ingesting such bacterial cells and harboring them as symbionts to utilize the oxygen and provide important metabolites such as amino acids and vitamins. Occasionally though, such symbionts would generate enough ROS to kill the host eukaryotic cell and live outside. Since such a situation was considerably detrimental to the hosts, over the next billion years or so, the bacterial symbionts had to give up many of their essential genes, transferring some to the host nucleus, and thereby became obligatory endosymbionts and finally the eukaryotic organelle mitochondria. The mitochondria are thus the energy store house of the eukaryotic cells, harboring both the electron transport pathway and the machinery for ATP synthesis. The release of important redox proteins such as cytochrome c or AIF from the mitochondria thus signals the cell of an impending energy catastrophe which prompts the cell to initiate the apoptotic event for committing a programmed form of suicide. The cells, however, appear to have evolved different mechanisms for initiating the apoptotic process. Mitochondrial cytochrome c in the cytosol acts through apoptosome formation, AIF acts directly on the nucleus, and azurin appears to act through complex formation and stabilization of p53, which then initiates mitochondrial cytochrome c release to the cytosol and caspase cascade activation (Figure 2). Since mitochondria have prokaryotic ancestry and the present day bacteria such as *P. aeruginosa* release redox proteins for triggering mammalian cell apoptosis, it appears that such a process has been evolutionarily conserved[50]. It would be of great interest to know if other bacteria may secrete other redox proteins that will enter mammalian cell cytosol and trigger their cell death, perhaps through yet another mechanism involving complex formation with a different cytosolic protein.

4. AZURIN IS AN ANTICANCER AGENT ACTIVE AGAINST HUMAN MELANOMA AND BREAST CANCER

Azurin has been reported not only to induce apoptosis in macrophages but also in human melanoma cells (UISO-Mel-2) harboring a functional tumor suppressor protein p53. The extent of apoptosis was significantly less when a p53-null mutant cell line (UISO-Mel-6) was used[62]. Azurin was shown to be

internalized in UISO-Mel-2 cells and was localized predominantly in the cytosol and in the nuclear fraction. In the p53-null UISO-Mel-6 cells, azurin was localized primarily in the cytosol, suggesting that intracellular trafficking of azurin to the nucleus was p53-dependent. Because azurin is known to form a complex with p53, thereby stabilizing it, the entry of azurin in the UISO Mel-2 cells raised the intracellular level of p53, thereby enhancing trafficking of Bax to the mitochondria and causing release of mitochondrial cytochrome c into the cytosol. This then led to the initiation of apoptosis, since p53 is a well-known inducer of apoptosis. Most interestingly, intraperitoneal injections of 0.5 mg of wt azurin to a group of immunodeficient nude (athymic) mice harboring melanoma tumors allowed tumor regression to the extent of 59% in 22 days. Multivariate analysis based on a random coefficient model showed that the difference in the tumor volume between treated and control animals was statistically significant[62]. As opposed to the wt azurin, the M44KM64E mutant azurin, deficient in complex formation at the N-terminal and stabilization of p53, exhibited much less cytotoxicity toward the UISO Mel-2 cells, suggesting a role of azurin-mediated p53 complexation and stabilization in cancer cell apoptosis and regression[62].

More recently, the entry of azurin into the human breast cancer cell line MCF-7 cells with consequent induction of apoptosis has been reported[4]. Similar to the human melanoma cell line UISO-Mel-2, azurin was shown to traffic to the nucleus but unlike UISO-Mel-2 cells, azurin was also found in the mitochondria. While the p53-positive MCF-7 cells were highly susceptible to azurin-mediated apoptosis, the p53-negative mutant MDA-MB-157 cells were less susceptible. Similar to azurin action in UISO-Mel-2 cells, azurin treatment of MCF-7 cells allowed enhanced level of p53 in mitochondria, nuclear and cytosolic fractions, indicating a portion of p53 normally located in the cytosol or the nucleus is translocated to the mitochondria. Interestingly, the Bax level in azurin-treated MCF-7 cells also went up in the mitochondria. Similar translocation of azurin to the mitochondria. Such translocations presumably led to a disruption of mitochondrial membrane potential, since considerable amount of mitochondrial cytochrome c was found to be released into the cytosol. It is interesting to note that Punj *et al.*[4] found that azurin had less apoptotic effect in normal mammary epithelial cells such as HBL 100 and MCF-10F cells than the MCF-7 tumor cells, suggesting the tumor cells as preferential target sites. A clinically important finding of Punj *et al.*[4] was that azurin allowed significant regression of breast cancer in vivo in athymic mice when injected daily to a group of 10 mice each at 1 mg level, as compared to an untreated group of 10 mice; 22 days after the start of treatment, the mean tumor volume in treated mice was only 22% of the mean tumor volume for the control mice, demonstrating a 78% tumor growth inhibition. At the conclusion of the experiment on the 29th day, the mean tumor volume in the azurin-treated

group was only 15% of the mean tumor volume of the control group, an 85% reduction in tumor volume. Immunohistological examinations of the tumors from both the treated and control groups showed marked increase of apoptotic cells, disintegrated parenchyma, and a sharp reduction in the number of proliferating cells in the azurin-treated group as compared to the control.

The elaboration of a member of the cupredoxin family, azurin, to the outside medium by *P. aeruginosa* is an interesting example of the potential of microorganisms in the treatment of cancer[14]. Live bacteria such as *Clostridium novyi, Salmonella typhimurium, Mycobacterium bovis* etc., have been used in an effort to fight tumor progression in experimental animals[14] and *M. bovis* has found significant use in the treatment of human bladder cancer. Genetically improved, attenuated strains of *Salmonella*, that can target tumor cells preferentially at about 1000 : 1 than normal cells, have been used as vectors to introduce specific enzymes such as cytosine deaminase to the tumors. Cytosine deaminase converts a nontoxic prodrug 5-fluorocytosine to a highly toxic anticancer drug 5-fluorouracil. Thus injection of such attenuated *Salmonella* to mice harboring lung tumors and subsequently given injections of 5-fluorocytosine has been reported to allow significant reduction in the size of the tumors (Figure 3). Use of live bacteria, even when attenuated, however, leads to problems of toxicity and side effects, thus limiting the usefulness of live bacteria in the treatment of cancer[14]. The more promising area of investigation, therefore, appears to be the use of bacterial products as potential anticancer agents. For example, bacteria such as *Sorangium cellulosum*, a myxobacterium, have been reported to produce secondary macrolide metabolites epothilone A and epothilone B which are microtubule depolymerization inhibitors. Cellular microtubules represent the molecular targets of Paclitaxel (taxol) isolated from the Pacific yew tree which has been widely used clinically in the treatment of a variety of solid tumors of breast, ovary, lung and colon[3, 16]. Epothilones have several advantages over taxol. They are more water soluble than taxol, which is advantageous for formulations since some of the ingredients in taxol formulation cause major side effects. More importantly, the epothilones are better able in inhibiting the growth of tumor cells overexpressing the P-glycoprotein, thus signifying that epothilone-like compounds may be useful in the treatment of multidrug resistant tumors, something that is problematic with taxol. Many epothilone analogs have been synthesized and some such as Desoxyepothilone B are more promising because of their efficacy against a range of tumors and relative lack of toxicity[16].

While the myxobacteria produce macrolides as anticancer agents, *P. aeruginosa* and *B. cepacia* produce low molecular weight, soluble cupredoxins that are potential anticancer agents (Figure 3). The involvement of a hydrophobic

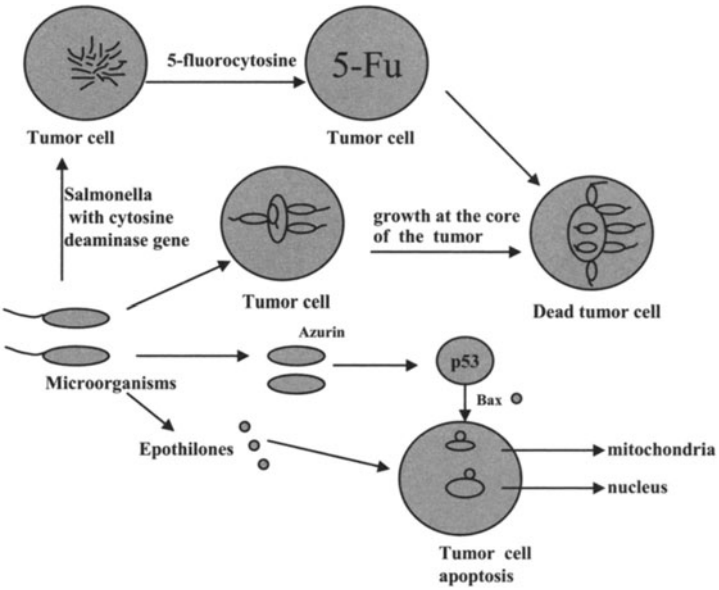

Figure 3. Various microbial pathways for the induction of cancer cell death. Microorganisms such as *Salmonella* can enter tumor cells and thrive in the anaerobic core of the tumor, thus preventing nutrient supply in surrounding cancer cells, causing their death and cancer regression. Attenuated *Salmonella* cells producing cytosine deaminase have been used as vectors which preferentially target the tumor cells. When 5-fluorocytosine, a nontoxic prodrug, is injected in the body, the prodrug is preferentially converted to the highly toxic 5-fluorouracil (5-Fu) in the tumors, causing their death. Microorganisms such as *P. aeruginosa* secrete azurin which triggers cancer cell death through complex formation and stabilization of p53. Myxobacteria such as *Sorangium cellulosum*, on the other hand, produce secondary metabolites such as the macrolide epothilone A and epothilone B, which along with their chemically modified derivatives, have been shown to have strong anticancer activity against a range of tumors.

patch present in the cupredoxins appears to be important not only for its interaction with the partners in the electron transfer pathway[18], but also for its interaction with the tumor suppressor protein p53. For example, when two Met residues in the hydrophobic patch of azurin were replaced by polar amino acids (M44KM64E), the resultant protein with the distorted hydrophobic patch could not form oligomeric complexes with p53 and did not allow significant stabilization of p53[25, 61]. The stabilization of p53 by wt azurin is believed to be due to its complex formation at the N-terminal of p53 where Mdm2 is known to bind at amino acid region 13–27 on p53 and enhances p53 ubiquitination and proteolytic degradation[38]. It is, however, not known if the mutant M44KM64E azurin may bind at the C-terminal of p53, which is involved in

p53 oligomerization[48]. Glycerol gradient experiments described by Yamada *et al.*[61] and Goto *et al.*[25] demonstrated reduced oligomerization of p53 when p53 was incubated with the M44KM64E mutant protein. If the reduced p53 oligomerization is due to complex formation with mutant azurin, either stable or transient, at the C-terminus, it would be of great interest to know whether such a complex may activate processes other than the induction of apoptosis such as an effect on cell cycle arrest, which is known also to be a function of disruption of p53 oligomerization[48].

5. CONCLUDING REMARKS

Enzymes that are involved in ATP metabolism or its conversion to other adenine nucleosides or nucleotides in cellular energetics are intracellular. Similarly, redox enzymes such as cupredoxins that are normally involved in electron transfer pathways are also intracellular. However, ATP-utilizing enzymes are also located on external surfaces of plasma membranes in mammalian cells where they modulate the level of adenine or uracil compounds and thereby control the extent of purinergic receptor activation[67]. Prokaryotes such as *P. aeruginosa* or *B. cepacia* have also recently been shown to secrete such ATP-utilizing or redox enzymes to the outside, particularly during growth in presence of eukaryotic proteins and perhaps during infection of mammalian host cells. Such secreted enzymes modulate mammalian cell functions including their cell death by acting on the host cell surface-associated purinergic receptors or by entering the host cells and forming complexes with host proteins such as p53 which in turn control the trafficking of the bacterial protein to various organelles. A protein such as p53 plays pivotal roles in many cellular functions in the host including cell cycle progression and cell death. The secreted bacterial proteins can then modulate such vital host functions through stable or unstable associations. As we know more about the critical domains of bacterial proteins such as azurin that interact with various domains of p53 and thereby modulate its various functions, it should be possible to develop short synthetic peptides that can be used as drugs to enhance preferential killing of cancer cells. Alternatively, since many of the bacterial enzymes are involved in triggering phagocytic cell death, it should be possible to screen or rationally design inhibitors of such enzymes that can be used as anti-infective drugs in the treatment of bacterial infections.

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3

IRON REGULATION AND SIDEROPHORE SIGNALLING IN VIRULENCE BY *PSEUDOMONAS AERUGINOSA**

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1. INTRODUCTION

Iron is an essential nutrient for almost all forms of life, due to its fundamental role in the biochemistry of all cells, including bacteria. Although abundant on the Earth's surface, iron is scarcely bioavailable because of its poor solubility in aqueous environments under aerobic conditions[119]. At neutral pH in the presence of oxygen, ferric iron [Fe(III)] precipitates in water solution as oxy-hydroxide polymer, and the concentration of free (i.e., assimilable) Fe(III) in equilibrium with the oxy-hydroxide form is $\approx 10^{-9}$ M. This implies that, under physiological conditions, the concentration of soluble iron obtained by passive diffusion through the membrane(s) is far below the threshold ($\approx 10^{-6}$ M) required to support the bacterial demand[18]. Bacterial parasites which multiply within higher organisms are also faced with the problem of obtaining iron from the host. In mammals, most iron is sequestered intracellularly

*Dedicated to Prof. N. Orsi, who gave me personal guidance and direction throughout my scientific career, with immense gratefulness and affection.

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by haem, iron-sulfur clusters and ferritins, while in the body fluids it is kept in solution by carrier proteins, such as transferrins and lactoferrins, which have an extremely high affinity for Fe(III)[119]. Although essential for nutrition, iron can also have deleterious effects if supplied in excess to living organisms. Intracellular Fe(II) catalyses the formation of reactive oxygen species (hydroxyl and oxygen radicals) in the presence of hydrogen peroxide (through the Fenton and Haber–Weiss reactions, respectively), which can damage proteins, lipids and nucleic acids[24].

To reconcile the nutritional requirements with the need to keep iron below the hazardous level, bacteria have evolved sophisticated strategies to control iron uptake. Among these, the coordinate synthesis of low-molecular weight Fe(III) chelating compounds, called siderophores (from the Greek σιδηρος = iron and φορεας = carrier), and their membrane transport systems provides a frontline solution to the problem of bacterial iron intake[53]. As a general rule, siderophores are synthesized in response to iron starvation (IS) and, once excreted in the medium, they can solubilise iron from insoluble precipitates, or extract it from complexing agents, delivering the metal to the cell through binding cognate receptor proteins located on the cell surface. Siderophore synthesis and uptake must be shut-off suddenly after restoration of intracellular iron homeostasis, since iron overloading would result in intoxication. Regulation of siderophore synthesis depends primarily on the activity of transcriptional repressors that sense the presence of sufficient intracellular iron and inhibit the transcription of siderophore synthesis and uptake genes. In recent years, however, elucidation of the mechanisms used by bacteria to procure iron has provided novel insights into the underlying regulatory circuitry. The Fur- and DtxR-like proteins of Gram-negative and Gram-positive bacteria accomplish the crucial function of halting iron uptake by repressing the transcription of numerous genes within their regulons, including those for siderophore synthesis and transport. When loaded with Fe(II), these repressors form homodimers that bind the operator sequences within their target promoters, thereby blocking transcription. Expression of nearly 100 genes was found to be regulated by Fur in *Escherichia coli*[60, 109], and a similarly impressive array of genes can be predicted to constitute the Fur regulon of the opportunistic pathogen *Pseudomonas aeruginosa*[102, 104, 148]. However, Fur- and DtxR-like proteins are not the only regulators of siderophore-mediated iron acquisition. At a lower hierarchical level, additional regulatory devices acting positively on the expression of iron uptake genes have been identified, including two-component transduction systems, AraC-like regulators of siderophore synthesis and transport, receptor-mediated signalling systems, and regulatory cascades involving extracytoplasmic function (ECF) sigma factors[30]. Remarkably, examples of all these regulatory strategies for iron uptake were recognized in *P. aeruginosa*[114, 154].

Sensing low iron levels not only results in the induction of iron uptake systems, but can also have an overall impact on the expression of virulence genes. Infectious bacteria, especially the opportunistic pathogens, must adapt themselves to very diverse environments during their life, and the ability to promptly respond to environmental changes assures a successful colonisation of different ecological niches. During infection, iron deficiency can be considered a leading signal communicating the entry of the bacterium into the mammalian host tissues, where the presence of transferrin and lactoferrin reduce iron availability[119]. Therefore, the ability of the bacterium to sense and counteract the iron withholding defence of the host will not only affect the onset of the infectious process (i.e., the ability of the bacterium to multiply in vivo), but also the expression of its virulence repertoire (i.e., the pathogenetic potential), thereby influencing the final outcome of the host–parasite interaction. In this respect, *P. aeruginosa* represents the paradigmatic example of a pathogen capable of selectively ensuring the expression of a certain subset of iron-regulated genes in response to the extremely diverse ecological niches it could occupy.

Pseudomonas aeruginosa is a worldwide distributed inhabitant of soil and moist environments, endowed with a wide metabolic versatility enabling it to survive in diverse habitats, including the mammalian host[108]. In fact, *P. aeruginosa* is a major opportunistic human pathogen capable of infecting injured, burned, immunodeficient, or otherwise compromised patients[86]. It is also a leading cause of severe pulmonary damage in cystic fibrosis patients[33]. Due to its typical respiratory metabolism and wide trophic niche, it can reasonably be assumed that *P. aeruginosa* would require a considerable amount of iron for growth. Therefore, it is not surprising that *P. aeruginosa* has evolved the striking ability to capture iron through a variety of strategies, including the synthesis of multiple siderophores, the uptake of several heterologous siderophores and iron chelates of bacterial and fungal origin, and the utilisation of haem and haemoglobin. The iron uptake systems of *P. aeruginosa* have recently been reviewed[114] and their description is out of the scope of this contribution. This chapter will address the mode of siderophore synthesis regulation and uptake in *P. aeruginosa*, as well as the molecular mechanisms enabling siderophore receptors to trigger the expression of functionally linked genes. An increasing body of evidence indicates that iron uptake, siderophore signalling, and transcriptional control of certain virulence factors implicate an integrated regulatory process.

2. IRON REGULATION IN *P. AERUGINOSA*: THE MASTER ROLE OF FUR

Interest in the genetics and biochemistry of iron-repressible gene expression in *P. aeruginosa* was initially stimulated by the evidence that the production

of exotoxin A, an ADP-ribosylating toxin produced by pathogenic *P. aeruginosa* strains, was reduced in high-iron media, reminescent of the regulation of siderophore synthesis (reviewed in refs [55, 148]). Later, it became evident that a unifying logic underlies the different aspects of iron-regulated gene expression in pathogenic bacteria, including *P. aeruginosa*, and that siderophores synthesis and the production of various virulence-related exoproducts are intimately linked processes[84].

In Gram-negative bacteria, transcriptional repressors which belong to the Fur family act on top of the hierarchy of iron-controlled gene expression[60]. Despite the attention given by many groups to iron regulation in *P. aeruginosa*, Pa-Fur was identified only in 1991, that is, 10 years after the discovery of *E. coli* Fur[59, 118]. This delay can be ascribed to the fact that Fur is essential in *P. aeruginosa* (and few other species), and its mutation can have lethal effects[148]. *P. aeruginosa* and the *E. coli* Fur also differ at the genetic level, given that the *P. aeruginosa fur* gene is neither autoregulated nor does it undergo Crp (catabolite repression) control as does the *E. coli* counterpart. Moreover, the transcription of the *P. aeruginosa fur* gene is rather complex, being directed by tandemly arranged promoters, one of which lies in the divergently oriented *omlA* gene. The downstream promoter contains a typical consensus for RpoD-dependent RNA polymerase (RNAP), while the upstream promoter has no obvious sequence signature, raising the possibility that additional regulatory proteins could be required for transcription arising from this promoter[103].

Purified Pa-Fur was used as an affinity chromatography ligand to select *P. aeruginosa* sequences carrying a functional Fur-binding site. This approach made it possible to retrieve a minimum set of 24 genes or operons likely transcribed from Fur dependent promoters from the *P. aeruginosa* genome, and to define a consensus sequence for the *P. aeruginosa* Fur box[102]. As for *E. coli*, the Pa-Fur box showed the canonical palindromic sequence GATAAT-GATAATCATTATC (matching residues are underlined), a feature reflecting the similarity between *P. aeruginosa* and *E. coli* Fur operator regions[102]. These are constituted by one to three overlapping or tandemly arranged Fur boxes that provide an extended pattern of protection in DNAse footprinting assays[10, 102, 148].

2.1. The Pa-Fur Architecture

Pa-Fur is a 135 amino acid protein whose fold is composed of two domains, the N-terminal domain (residues 1–83) implicated in DNA binding, and the C-terminal domain (residues 84–135) responsible for homodimerisation (Figure 1, panel A)[112]. The DNA-binding domain consists of four α-helices (H1–H4) followed by an antiparallel two-stranded β-sheet. H4 and the loop between H1 and H2 helices are implicated in DNA recognition. The

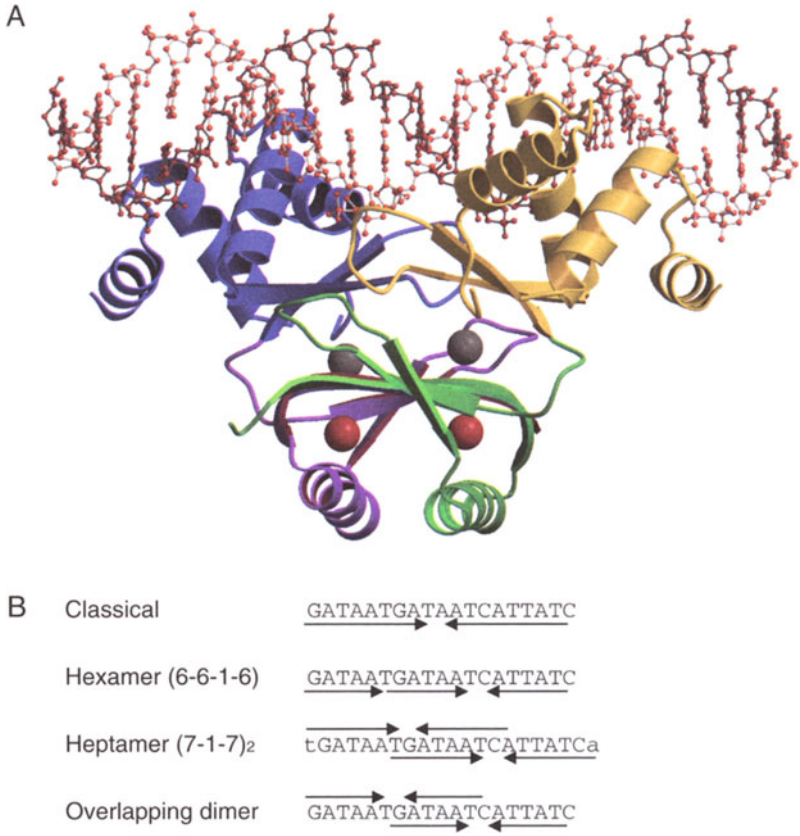

Figure 1. Model of Fur-DNA complex formation. A. *In silico* modelling of Pa-Fur binding to DNA. Ribbon diagram of the DNA-bound Pa-Fur dimer. Each Pa-Fur monomer is composed of two major domains (blue and violet in the left monomer; ochre and green in the right monomer). Four α-helices and an antiparallel two-stranded β-sheet (blue in the left monomer; ochre in the right monomer) constitute the DNA-binding domain. The dimerisation domain is prevalently an α/β structure (violet in the left monomer; green in the right monomer). Two metal coordination sites were recognised in each monomer. Site 1 is entirely located in the dimerisation domain and represents the putative Fe(II)-binding regulatory site (iron atoms are in red). Site 2 forms a bridge between the dimerisation domain and the DNA-binding domain, and was suggested to be the structural Zn(II) binding site (zinc atoms are in black). The figure was produced as described in ref. [112]. The protein structure was manually docked to the canonical B-DNA containing the *pvdS* operator sequences. Model by courtesy of Dr. E. Pohl, EMBL, Hamburg, Germany. B. Comparison of different interpretations of the Fur box. According to the classical interpretation, the Fur box is defined as two 9-bp inverted repeats spaced by a single nucleotide[37]. The same sequence can also be interpreted as an array of three $NAT^{A}/_{T}AT$ hexamers (6-6-1-6 arrangement; ref. [45]). Alternatively, the Fur box can be conceived as two overlapping inverted repeats. In the $(7\text{-}1\text{-}7)_2$ model the combination of inverted heptamers would define a 21-bp sequence (the extra nucleotides at the 5′ and 3′ termini are in lower case; ref. [9]). In the overlapping-dimer binding model, two inverted heptamers separated by one G:C are tandemly arranged to encompass the 19 bp Fur box[78]. According to both the $(7\text{-}1\text{-}7)_2$ and the overlapping-dimer binding models, a single Fur dimer would bind to each inverted repeat on opposite sides of the DNA helix.

dimerisation domain is prevalently an α/β structure in which one long α-helical region (H5) is juxtaposed to three antiparallel β-strands. Pa-Fur was crystallised in the presence of zinc and two coordination sites for Zn(II) were recognised in the molecule. Site 1 is entirely located in the dimerisation domain and can exchange Zn(II) with Fe(II), while site 2 forms a bridge between the dimerisation domain and the DNA-binding domain, and it is likely to represent the structural Zn(II) binding site. Different from *E. coli* homologue, Pa-Fur does not contain the C-terminal Cys-X_4-Cys motif which is implicated in the binding of the third Zn(II) atom in *E. coli* Fur[52]. Site 1 represents the putative regulatory site; binding of Fe(II) to this site is predicted to induce a conformational change of the DNA-binding domain in the Pa-Fur dimer, rendering the repressor competent for DNA recognition[112].

At present, however, the molecular basis for DNA recognition by Fur is still controversial. Since the mid-1980s, a multiplicity of experimental approaches have been used to define the basic Fur binding site, and at least four models of Fur–DNA interaction have followed one upon the other (Figure 1, panel B). It was initially proposed that the Fur dimer recognises the 19-mer consensus as 9-bp inverted repeats separated by one nucleotide[37]. However, proteins that bind to DNA by the classical helix-turn-helix motif are predicted to require shorter (*c.* 12-bp) operator sequences[63], and the small size of dimeric Fur could not account for the extended pattern of DNA recognition deduced from several footprinting experiments. In an attempt to solve this incongruency, it has been proposed that the Fur box can be regarded as an array of adjacent $NAT^{A}/_{T}AT$ repeats, and that three of these hexameric motifs, two in forward orientation and one in reverse orientation (6-6-1-6 arrangement), are the minimum requirement for initial operator binding by *E. coli* Fur[45]. In a recent revision of this model, the Fur box was reinterpreted as two overlapping inverted repeats, each one containing an internal G:C spacer (Figure 1, panel B). According to the heptameric $(7\text{-}1\text{-}7)_2$ arrangement, the Fur box would be redefined as a 21-bp sequence carrying an extra T at the 5′ and an extra A at the 3′[9]. Alternatively, the overlapping-dimer binding model predicts the unit of interaction with the single Fur dimer to be composed of two inverted hexamers separated by a single G:C base pair, with two units encompassing the whole 19-bp Fur consensus[78]. In the last two models, two Fur dimers are predicted to recognise sequences offset by 6 bp, and are therefore envisioned to bind DNA on opposing faces. Such an arrangement has also been observed in the crystal structure of the DtxR-operator DNA complex[158]. While studies of Pa-Fur binding to *P. aeruginosa* operator sites are not yet conclusive, the similarities observed with the *E. coli* homologue at both the protein and operator level argue for an overall conserved mechanism of DNA recognition. In fact, the model presented in Figure 1 (panel A) fits well with the docking of the two H4 helices within the major groove of a canonical

B-DNA bearing Fur-binding site, with the loops between the H1 and H2 helices also being implicated in DNA recognition[112]. This model also supports the view that two Pa-Fur dimers recognise 5-bp spaced sites located on opposite sides along the DNA sequence, in fair agreement with the revised topology and binding stoichiometry of the canonical Fur box.

2.2. The Pa-Fur Regulon: Not only Iron Transport Genes

Under physiological conditions, there are 10,000–40,000 copies of Pa-Fur in the cell, a much higher number compared with other repressors[148]. This could in part be due to the large number of genes that are regulated by Pa-Fur, or to its tendency to multimerise through protein–protein interactions, or the relatively low affinity of Fur for its operator sites[45]. Such a high physiological amount of the repressor could also be related to the iron scavenging properties of Pa-Fur, that would sequester any excess of iron inside the cell prior to incorporation into storage proteins. Remarkably, *fur* promoter mutants in which Fur is poorly expressed as the result of reduced *fur* transcription display an increased sensitivity to reactive oxygen species[148].

As expected, the majority of *P. aeruginosa* genes carrying a functional Fur-binding site in their promoter are directly implicated in iron transport[102]. Few genes, however, are distantly related to iron metabolism, while accounting for basic cellular functions. Notably, some encode alternative isoforms of iron-containing enzymes. The *fumC* gene, encoding a fumarase, is part of a Fur-regulated operon with *fagA*, *orfX* and *sodA*. Fumarases take part in the tricarboxylic acid (TCA) cycle and generally contain a catalytic iron-sulfur cluster; FumC, however, does not require iron for activity and can replace the iron-containing homologues, FumA and FumB, during IS. Similarly, SodA is a manganese-dependent superoxide dismutase implicated in the defence from the oxidative stress under conditions in which the iron-cofactored counterpart SodB would be inefficient because of reduced iron availability. Another Fur-regulated gene is *nuoA*, encoding a subunit of complex I (NADH:ubiquinone reductase) of the electron transport chain. Interestingly, the dependence of alginate biosynthesis on *fumC* and the indirect control of the *fagA-fumC-orfX-sodA* operon by AlgU suggested that both the iron level and redox status of the cell may also affect alginate production in *P. aeruginosa*. It was proposed that mucoid bacteria need more iron for electron transport proteins in order to generate enough ATP for alginate synthesis. By increasing the expression of FumC and NuoA under conditions of severe iron limitation, the reducing power [NAD(P)H and $FADH_2$] generated from the TCA cycle would still be conveyed into the electron transport chain to fulfil the ATP demand for the expensive alginate biosynthetic machinery[64]. Miscellaneous Fur-controlled genes included a homologue of the *sulA* gene

of *Serratia marcescens*, which is predicted to encode an inhibitor of the cell cycle implicated in slowing cell division under conditions of IS, the *tolQRAB* system, and a two-component regulatory system similar to the *Pseudomonas syringae* LemA sensor kinase [102, 148].

An intriguing aspect of iron regulation in *P. aeruginosa* is the relationship between Pa-Fur and the expression of the exotoxin A (*toxA*) gene. This issue has been reviewed elsewhere in this volume[55], and herein we shall only provide a concise outline of the iron-responsive components of the complex regulatory network controlling *toxA* expression. The *toxA* gene is transcribed from a single iron-regulated promoter which lacks canonical recognition sequences for RpoD-dependent RNAP, which would suggest the involvement of alternative sigma factor in *toxA* transcription[148]. At least five regulatory genes, namely *fur*, *pvdS*, *ptxR*, *regA*, and *vfr*, have so far been implicated in the transcriptional control *toxA*. A sixth regulatory gene, *ptxS*, acts indirectly on *toxA* expression through a repressor activity on the *ptxR* gene[26]. The Vfr activator protein, homologous to Crp of *E. coli*, positively affects exotoxin A expression but has no apparent effect on iron regulation. Conversely, expression of both *regA* and *ptxR* appears to be iron regulated, and the products of these genes are required for optimal *toxA* transcription. Although *regA* and *ptxR* genes differently modulate *toxA* expression[55], they display a quite similar pattern of transcription initiation and iron regulation[148]. Transcription of both *regA* and *ptxR* originates from a constitutive promoter during the early growth phase, while it is shifted to an iron-repressible promoter during the late logarithmic phase. Sequence analysis and protein–DNA interaction assays ruled out the direct binding of Pa-Fur to *toxA*, *regA*, or *ptxR* promoters, though the expression of all these genes was clearly iron repressible and significantly increased in a *fur* mutant background under certain growth conditions. Thus, in order to bridge the gap between *fur* and *regA* or *ptxR*, one (or more) intermediate(s) have to be added to the already complex hierarchy of *toxA* regulation. A major breakthrough in this field was achieved in 1996 with the observation that PvdS, an ECF sigma factor of the IS subfamily[32, 85, 95], was essential for exotoxin A production[100] as well as for transcription of both *regA* and *ptxR*[149]. The *pvdS* gene was originally identified as a positive activator for the production of the siderophore pyoverdine[32] (see Section 4.2), but was later shown to be the central regulator of at least 26 iron-repressible genes in *P. aeruginosa*[104]. *pvdS* is transcribed from a typical RpoD-dependent promoter encompassing a single Fur box with 79% identity to the consensus 19-mer. PA-Fur binding to the *pvdS* promoter was observed both in vitro and in vivo. Coherently, transcription of *pvdS* is fully repressed under iron-sufficient conditions, while repression is relieved in a *fur*-defective background[10, 102]. As the result of Pa-Fur repression on *pvdS*, all the PvdS-dependent promoters, including those controlling *regA* and *toxA* transcription, are indirectly

repressed during *P. aeruginosa* growth in iron-rich medium[100]. Accordingly, neither *toxA* nor *regA* mRNAs are detectable in a Δ*pvdS* mutant[102].

Recently, the resolution of the Pa-Fur structure provided a basis to investigate some functional peculiarities of Pa-Fur, as inferred from the unusual phenotypes of Pa-Fur mutants. Replacement of His86, responsible for Fe(II) coordination, with either Arg or Tyr abrogated repression of siderophore synthesis but surprisingly had no effect on the pattern of exotoxin A expression[148]. The His86 mutant proteins have a reduced ability to bind the Fur boxes onto the *pvdS* promoter in vitro, consistent with constitutive siderophore production[10]. His86 is involved with Asp88, Glu107, and His124 in the octahedral coordination of Fe(II) ion, and its replacement can have obvious effects on metal regulation[112]. Another Pa-Fur mutant in which Ala10 was replaced with Gly was obtained from strain PAO6261[10], which carries a mutation in the *anr* gene, encoding a transcriptional regulator of the Fnr family[139]. This strain overproduced siderophores, and the manganese-cofactored superoxide dismutase SodA[10, 65]. Consistent with this phenotype, the mutated Pa-Fur purified protein retained partial or no binding activity to the Fur boxes located within iron-regulated promoters[10, 65]. Indeed, Ala10 is the first amino acid of a loop separating the adjacent H1 and H2 helices. Since this loop is predicted to be involved in DNA-binding activity, replacement of Ala into Gly might cause partial unfolding and loss of activity[112]. Intriguingly, the Ala-10-Gly mutant was conditionally constitutive for exotoxin A expression, that is, transcription of both *regA* and *toxA* genes was iron repressible under aerobic conditions, but constitutive under microaerobic conditions[10]. These data are difficult to interpret, since constitutive expression of the siderophore genes was detected under aerobic conditions. Given that *pvdS* expression is 10-fold reduced under microaerobic conditions[100], an explanation could be that exotoxin A expression follows a different route, possibly one governed by the *ptxR* gene product[56]. Indeed, *ptxR* expression under aerobic conditions was normally iron regulated in the Ala-10-Gly mutant, similar to what was observed in the wild type, while it was constitutive under microaerobic conditions[149].

3. THE RATIONALE OF COMBINING SIDEROPHORE TRANSPORT AND SIGNALLING: INSIGHTS FROM THE FEC SYSTEM OF *E. COLI* K-12 AND THE PUP SYSTEM OF *P. PUTIDA* WCS358

A hierarchical organisation of sensorial responses governs the expression of transport systems for exogenous iron chelates. First, the cell must sense the

iron-limited environment, then the presence of the exogenous iron carrier, which represents the extracytoplasmic stimulus. The information of iron chelate binding to its outer membrane receptor is transferred from outside to inside the cell, where it triggers the expression of cognate uptake genes. The cytoplasmic response occurs through the activity of regulators which activate specialised sigma factors of the ECF subfamily[85]. Since 1990s, two paradigmatic examples of siderophore-mediated signal transduction pathways were investigated in great detail: the ferric (di)citrate uptake (Fec) system of *E. coli* K-12 and the uptake system for heterologous pseudobactins BN7 and BN8 (Pup system) of *P. putida* WCS358. These model systems served as the reference for the investigation of mechanistically similar systems in *P. aeruginosa*, and for this reason they deserve an extensive description.

3.1. A Triad of Essential Partners

Unlike *Salmonella, E. coli* cannot use citrate as the sole carbon source due to the lack of a specific transport system. However, exogenous citrate can fulfill the nutritional iron requirement of *E. coli* grown under condition of iron deficiency, acting as a siderophore[49]. When the iron concentration is limiting (<1 μM) and citrate is present in large excess in the extracellular milieu (>100 μM), 1:2 Fe(III)-citrate complexes are formed and transported into the cell via a specific receptor protein and an energy-coupled multicomponent transport system.

Induction of the ferric dicitrate transport genes occurs through a specific mechanism referred to as "surface signalling"[15]. Ferric dicitrate, the inducer, does not need to enter the cell to activate the expression of cognate transport genes. The induction process starts with the interaction between ferric dicitrate and the outer membrane receptor protein FecA, an event which triggers a cascade of protein–protein and protein–DNA interactions leading to increased expression of *fec* (*fe*rric *c*itrate transport) genes. The whole induction process requires structural and functional interactions among three partners: information is transferred from the receptor (FecA), through a transmembrane transducer (FecR), to an ECF sigma factor (FecI) that finally directs transcription of transport genes (Figure 2, panel A). Dicitrate acts as iron carrier and co-inducer [when combined with Fe(III) atom], but not as cotransporter since it is not internalised. The whole strategy is certainly advantageous to the cell, as it saves energy otherwise required for the baseline expression of the transport system when the transporter is unavailable.

The *fec* gene system is composed of seven adjacent open reading frames in the same transcriptional orientation, namely *fecIRABCDE* (Figure 2, panel A). The first two genes have a regulatory function and encode the alternative sigma factor FecI and the signal transducer FecR, while the remaining five genes

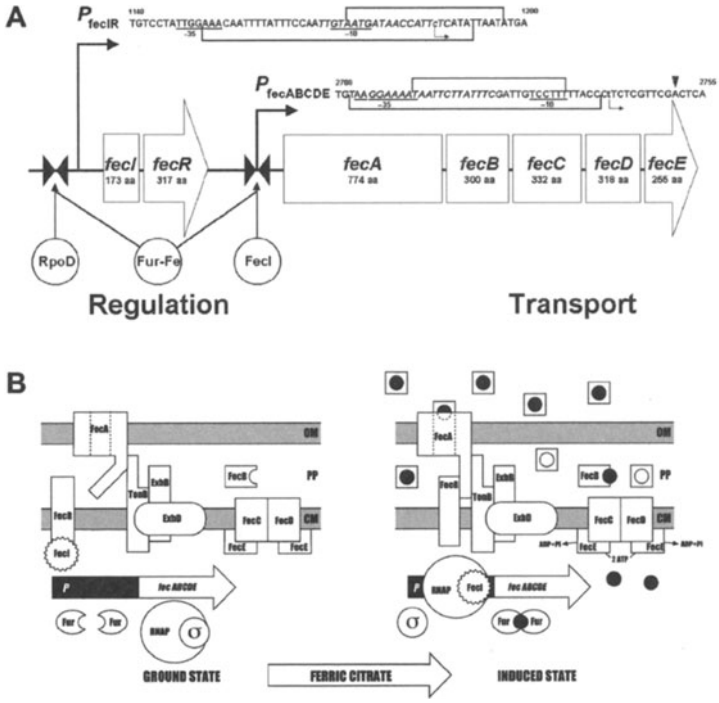

Figure 2. The ferric citrate uptake system of *E. coli* K-12. A. Physical organisation of the *fec* gene cluster and coregulation by Fur and FecI. Individual genes and corresponding protein products (aa are numbers of amino acids of unprocessed peptides) are shown in the boxes. The boldface bent arrows give the startpoints of *fecIR* and *fecABCDE* mRNAs and the direction of transcription. Fur boxes are represented as converging triangles. Nucleotide sequences of the *fecIR* (P_{fecIR}) and *fecABCDE* ($P_{fecABCDE}$) promoters are given on the upper part of the figure. Numbers above the first and last nucleotides correspond to positions in the published sequences[42]. The −10 and −35 promoter regions are underlined. Transcription start points are in lower case and the direction of transcription is indicated by downward bent arrows. The italicised sequence regions map the predicted Fur boxes. Horizontal square brackets delimit the experimentally confirmed Fur-binding regions[5] on the coding (upper brackets) and non-coding (lower brackets) DNA strands. The inverted triangle on the $P_{fecABCDE}$ sequence marks the critical nucleotide of the 3′-element affecting FecI binding and promoter activity[6]. B. Schematic of the Fec transport and regulatory proteins. Under iron-restricted conditions, transition from the ground state to the induced state occurs through binding of ferric citrate (boxed circle) to the FecA outer membrane receptor. The signal is transduced to the ECF sigma factor FecI through the response regulator FecR, and involves ligand-induced conformational changes of both FecA and FecR. Activated FecI recruits RNAP on the *fecABCDE* promoter (*P*) and directs transcription of *fec* transport genes. Transport occurs through transfer of iron from citrate to the periplasmic iron-binding protein FecB, then to the membrane-anchored FecCDE complex which translocates the metal into the cytoplasm at expenses of ATP hydrolysis. Both transport and signalling require an energised state of the cytoplasmic membrane and the TonB-ExbB-ExbD system. When sufficient iron is present, the system is silenced through the binding of the iron-loaded Fur repressor to the promoters of *fec* regulatory and transport genes. OM, outer membrane; PP, periplasmic space; CM cytoplasmic membrane.

encode transport proteins. Ferric dicitrate is translocated into the periplasm through the combined action of FecA, TonB, ExbB, and ExbD proteins, at the expenses of the protonmotive force. The electrochemical potential of the cytoplasmic membrane generates an activated form of TonB[96] which, in turn, drives a conformational transition of FecA to a transport-competent state, with the opening of the intramolecular channel for the passage of ferric dicitrate into the periplasm[71]. FecB is the periplasmic binding protein for ferric dicitrate. Three additional proteins of the ATP binding cassette (ABC) superfamily, namely FecC, FecD, and FecE, complete the transport pathway providing the device for the ATP-dependent translocation of iron across the cytoplasmic membrane[141].

The *fec* system is composed of two iron-repressible transcriptional units which are coordinately expressed in response to ferric dicitrate induction[42] (Figure 2, panel A). The upstream unit encompasses the *fecIR* regulatory genes and originates from an RpoD-dependent promoter (P_{fecIR}). The *fecIR* promoter contains a Fur box and does not require ferric dicitrate induction for baseline activity[42]. A second promoter (P_{fecABCDE}) is located downstream of *fecR*, and directs the synthesis of two mRNA species: a longer transcript encoding for all five transport proteins (FecABCDE) and a shorter one encoding FecA only. P_{fecABCDE} is endowed with structural characteristics of promoters recognised by alternative sigma factors of the ECF sub-family[85] and contains a Fur binding site. Thus, under iron-replete conditions, the Fe(II)-loaded Fur repressor blocks transcription of both *fecIR* and *fecABCDE* gene clusters. The presence of Fur-binding sites on both P_{fecIR} and P_{fecABCDE} provides the cell with a stringent and energetically advantageous control device for the uptake of citrate-bound iron. Under conditions of limiting iron, Fur cannot bind P_{fecIR} and P_{fecABCDE}, and the *fec* system is expressed at the baseline level. The FecI protein is activated following ferric dicitrate binding to FecA, leading to the expression of the *fec* transport genes and, by consequence, to an active influx of iron into the cell (Figure 2, panel B). When the intracellular iron homeostasis is restored, transcription of *fec* genes is promptly shut off by Fur binding to both P_{fecIR} and P_{fecABCDE}. By this way, a sudden block of *fec* transport genes anticipates the complete decay of the FecI sigma factor.

3.2. The Signal-Sensing Siderophore Receptor, FecA

A typical feature of surface signalling is that the outer membrane receptor is required not only for transport of the metal chelate, but also for induction of transport genes (Figure 2, panel B). This double function reflects the primary structure of all signal transducing receptors. In particular, FecA differs from bacterial siderophore receptors which lack the signalling function due to the presence of an approximatively 40-amino acid extension (FecR box)

at the N-terminus[48, 75]. As other siderophore receptors, FecA is composed of a β-barrel of 22 antiparallel β-strands, closed by a globular (plug) domain[46]. Structural data indicate that binding of ferric dicitrate causes major structural modifications of FecA, notably the gating of the substrate-binding pocket by surface loops and a conformational transition of the periplasmic region. The N-terminal domain of FecA could not be resolved due to its flexibility, but there is convincing genetic and biochemical evidence for its implication in recognition of TonB and signalling via FecR[17]. The TonB protein is anchored to the cytoplasmic membrane with the N-terminal region which contains a histidine residue (His20) that can be protonated thereby converting the protein to an energized state. The rest of TonB is exposed to the periplasm and can interact with FecA. Recognition involves the 160 region of TonB and residues 80–84 (TonB box) of mature FecA. Recent data indicate that the ferric dicitrate-induced conformational transition of the N-terminal domain of the FecA facilitates the interaction with TonB, and that the structure rather than the sequence of the TonB box is important for FecA activity[105].

The regulatory role for FecA was initially inferred by the observation that transport of dicitrate-bound iron from the periplasm to the cytoplasm occurs in a *fecA* null mutant, expressing the *fecBCDE* genes, while induction of *fecA* expression does not[62]. This is because ferric dicitrate (434 Da) can diffuse into the periplasm through the porin channels, making transfer of iron to FecB possible. The bifunctional activity of FecA was supported by the analysis of *fecA* mutants in which ferric dicitrate transport was uncoupled from the induction of *fec* genes and confirmed by genetic dissection of the protein. The N-terminal region of FecA (residues 1–127) is involved in the induction of the *fec* system and recognition of the TonB protein. Mutations in the putative TonB box of FecA abolished both induction and transport functions[15, 62], while a deletion encompassing the N-terminal extension (residues 14–68) affected induction only[71]. Therefore, induction and transport involve distinct, but probably related, conformational transitions of FecA[71]. This makes sense, since binding ferric dicitrate should convert FecA to the induction-active state, thereby switching on the self-sustained loop which leads to the amplification of all transport components encoded by the *fecABCDE* gene cluster.

3.3. The Transmembrane Transducer, FecR

FecR protein is the second component of the signal transducing pathway, and accounts for the transfer of the information from ferric dicitrate-loaded FecA to FecI in the cytoplasm. FecR is a 317-amino acid protein; the first 84 residues are located in the cytoplasm, residues 85–100 constitute the membrane-spanning domain, while the rest of the protein spans the periplasm[99]. Signalling involves physical interactions between the (periplasmic) N-terminal

extension of FecA and the C-terminus of FecR, as well as between the N-terminus of FecR and the (cytoplasmic) sigma factor FecI. Genetic and biochemical evidence supports this model. Point mutations in *fecR* may either reduce or abrogate transcription of *fec* transport genes, or cause their constitutive expression[99, 165]. FecR derivatives devoid of their C-terminal (periplasmic) region no longer respond to induction by ferric dicitrate but constitutively express the *fec* transport genes. Thus, the N-terminal region of FecR is sufficient for FecI activation and constitutive expression of *fec* genes[99]. The vectorial interaction FecA→ FecR→ FecI was demonstrated both in vitro by means of co-purification assays with individual tagged components, while protein domains responsible for the interaction of FecA with FecR and FecR with FecI were precisely mapped in vivo using a two-hybrid system. Two main interacting domains were identified in FecR: a short 1–85 region which recognises residues 1–173 of FecI, and a long 101–317 region which recognises residues 1–79 of FecA[44].

Two models can be proposed for signal transduction from the outer membrane receptor FecA to the cytoplasm. The first implies that the N-terminal extension of FecA dynamically interacts with the C-terminus of FecR in the periplasm, but the induction-productive pattern of interaction results from the allosteric transition of FecA consequent to ferric dicitrate binding. Alternatively, if it is assumed that FecR stably interacts with FecA and FecI even in the absence of induction, signal transduction could be mediated by a piston-like motive force resulting from structural changes (compression/extension) in FecA following ferric dicitrate binding, as it has been shown for bacterial chemoreceptors[107]. These structural rearrangements are then transmitted from the C-proximal domain of FecR through the inner membrane into the cytoplasm, where the N-proximal region of FecR converts FecI to the transcription-active state (Figure 2, panel B).

3.4. The ECF Sigma Factor, FecI

FecI is an 173 amino acid protein which shares substantial sequence similarity with members of the ECF subfamily of alternative sigma factors. *fecI* is cotranscribed with *fecR* and the two genes are translationally coupled, facilitating the interaction between the C-proximal domain of the FecI and the N-terminal region of FecR[147]. FecI is active on the P_{fecABCDE} promoter and, when overexpressed, it directs transcription of *fec* transport genes even in the absence of ferric dicitrate induction and FecR. However, ferric dicitrate-inducible transcription at the P_{fecABCDE} promoter requires the coexpression of *fecIR*, indicating that in vivo FecI is converted into a more active conformation by FecR[6, 147]. When combined with the core fraction of *E. coli* RNAP, FecI mediates the binding of RNAP to P_{fecABCDE} and directs transcription of

templates containing $P_{fecABCDE}$[6]. FecI selectively recognises $P_{fecABCDE}$; no other sigma subunit of the *E. coli* RNAP is endowed with such a narrow range of promoter specificity. FecI interaction with $P_{fecABCDE}$ involves the recognition of the non-conserved −10 and −35 regions and is dependent on a downstream regulatory element centred around position +11 relative to the start point of the *fecABCDE* transcript[5, 6, 42, 43] (Figure 2, panel A). The recognition of 3′-downstream sites is uncommon among RpoD-like sigma factors, although it has been reported for RpoE, RpoS, and ECF alternative sigmas[90, 106, 164].

Like all ECF sigma factors, FecI lacks almost the entire region 1, which in RpoD prevents promoter binding by region 4[39]. Nevertheless, FecI does not associate with $P_{fecABCDE}$ in the absence of core RNAP[6], and can be deprived of the 1.2 region with no effect on transcriptional activity[142]. Regions 2.1 and 4.2 of FecI are implicated in FecR-dependent activation[142], while the C-terminal 4.2 region (amino acids 139–158) contains a helix-turn-helix element typical of DNA-binding proteins[147]. Substitutions within the surface-exposed residues of the two helices affect FecI activity by altering the affinity of FecI for $P_{fecABCDE}$ and, consequently, the response of FecI to FecR-dependent activation[142]. A number of brilliant experiments made it possible to identify critical residues for FecI recognition by FecR[142]. Activation-defective *fecR* mutants were suppressed by substitution at position 12, 15, and 20 of FecI, though no allele specificity was observed for the *fecI* suppressor mutants. Using a two-hybrid system, direct interaction was observed between the cytoplasmic domain (residues 1–85) of FecR and wild-type or mutated FecI, while deletion of the 2.1 or 4.2 region of FecI abrogated the interaction with FecR[44, 142]. The interacting regions of FecI and FecR may therefore be confined to relatively short segments of FecR (residues 13–50) and to regions 2.1 and 4.2 of FecI. Then, how can a structural transition of FecR activate FecI-dependent transcription? By analogy with the regulatory mechanism of RpoE[2], it can be hypothesised that FecI is synthesised in an active form which promptly associates with inactive FecR as a result of translational coupling. While bound to inactive FecR, FecI is transcriptionally inert and perhaps susceptible to controlled proteolysis. This explains why FecI and FecR are both expressed under low iron conditions, but transcription from $P_{fecABCDE}$ occurs at a basal level without ferric dicitrate induction. When ferric dicitrate is present, signalling of ferric dicitrate binding to FecA results in secondary structure transition in the cytoplasmic domain of FecR. Activated FecR releases active FecI, which should rapidly be sequestered by RNAP for transcription of *fec* transport genes, leading to amplification of both signalling and transport pathways[142]. Remarkably, FecR has recently been shown to promote FecI binding to the β′ subunit of RNAP and to protect FecI from proteolytic cleavage[89]. This feature also explains how FecI can successfully compete with the major sigma factor RpoD for core RNAP binding. During exponential

growth, a single *E. coli* cell is predicted to contain *c.* 2,000 RNAP molecules. Of these, *c.* 700 are not engaged in transcription cycles[70], resulting in being accessible to competitive binding by an excess (*c.*, 200 molecules) of different sigma factors which are normally present in the cytoplasm. The basal level of FecI in repressed cultures (i.e., in LB medium without citrate) is less than 1 molecule per cell compared with *c.* 700 molecules of RpoD[88]. Even if FecI concentration is expected to increase dramatically following derepression, the unfavourable core binding affinity of FecI (>6-fold lower) compared with that of RpoD makes the replacement of RNAP-associated RpoD by free FecI questionable[87]. However, in the cell architecture, the ratios of RNAP-bound individual sigma factors is locally variable, and the physical association of *fecI* with $P_{fecABCDE}$ is expected to result in a high density of the *fecI* product near its target promoter. Moreover, once the FecR–FecI complex is formed, activation of FecR could increase the affinity of FecI for the core-RNAP[89] and/or for the cognate promoter, thereby facilitating assembly of the FecI–RNAP complex and promoter binding.

3.5. Surface Signalling in *P. putida* and Related *Pseudomonas* spp.

As a rule, the fluorescent pseudomonads not only produce multiple siderophores, but also have developed the ability to acquire a variety of exogenous iron chelates[114]. Fundamentally, the same logic regulates the different transport systems for exogenous iron chelates in *E. coli* and *Pseudomonas* spp., in the sense that full expression of uptake genes requires induction by the iron carrier. However, different mechanisms of signal transduction mediate the induction of uptake genes[30]. Historically, receptor-mediated siderophore signalling in fluorescent pseudomonads was unravelled by investigation of the *pupIR–pupB* system of *P. putida* WCS358[74]. The iron-regulated PupB protein is the outer membrane receptor for heterologous pseudobactins BN7 and BN8 (see Section 4 for details on pseudobactin structure). Similarly to FecA, PupB expression is induced by the heterologous pseudobactins BN7 and BN8[73]. The physical organization of the *pupIR–pupB* gene system is reminescent of the *fec* cluster. *pupIR* are cotranscribed from a single RpoD-dependent promoter which contains a putative Fur box, while the *pupB* receptor gene is separated from *pupIR* by an intergenic region containing the PupI-dependent promoter. Like FecA, the PupB protein contains an N-terminal extension (FecR box), which is responsible for signal transduction to the periplasm[12, 73]. PupR is 36.6% identical to response regulator FecR, and shows a similar location for the putative transmembrane spanning domain (positions 85–105). PupI is a 173-amino acid protein endowed with the structural features of iron-regulated ECF sigma factors involved in surface signalling[81]; PupI is 42.8% identical to FecI and contains a helix-turn-helix

element at similar position. Both *pupI* and *pupR* mutations cause a strong reduction of *pupB* expression irrespective of the presence of pseudobactin BN8. However, *pupB* expression is significantly lower in the *pupI* than in the *pupR* mutant background. In the latter case, overexpression of *pupI* strongly increases *pupB* expression but does not restore inducibility. Overexpression of both *pupI* and *pupR* in the *pupR* mutant reduces *pupB* expression in the absence of induction while it increases *pupB* expression upon induction. Therefore, the PupR regulator behaves both as an activator and as a repressor; it is essential in the induction process, but it prevents the PupI-dependent transcription of *pupB* in the absence of the inducer[74]. The antisigma activity of PupR reflects a general mechanism of negative control of ECF sigma factors by cognate regulators[69, 154], and constitutes the main functional difference between PupR and the FecR protein of *E. coli*. Mutations in either *tonB* or *pupB* abrogate pseudobactin BN8-mediated induction of PupB synthesis, suggesting that signalling requires both pseudobactin BN8 binding to PupB and an energy-coupled process mediated by the TonB protein, as in the case of the Fec system[13].

The transport of the homologous pseudobactin WCS358 in *P. putida* WCS358 is driven by an alternative receptor, PupA, whose expression is under the control of the PfrI and PfrA regulatory system[151]. The structural similarity between PupB and PupA and their different specificity facilitated the construction of chimeric receptors for recognition of the functional domains of the PupB protein. Analysis of uptake and signalling specificities of chimeric Pup receptors confirmed that the information for siderophore specificity and transport is contained within an extended C-terminal region of the receptor, while the signal-transducing domain is confined to its N-proximal region. A regulatory device involving signal transduction from the PupA receptor to the gene system for pseudobactin 358 biogenesis and uptake was initially postulated by Venturi and co-workers[151]. This hypothesis originated from two preliminary points. First, a positive effect of pseudobactin 358 on the expression of cognate biosynthetic genes was observed. Second, the presence of an N-terminal FecR box in PupA was suggestive of an interaction with a putative PupR-like response regulator. The IS sigma factor PfrI, which is essential for expression pseudobactin 358 biosynthetic genes, was initially regarded as the terminal candidate for signal transduction. However, PfrI is dispensable for the expression of the PupA receptor, and the *pfrI* gene is not physically linked to a putative response regulator gene[81, 150, 151]. Moreover, PfrI activity is dependent on PfrA, but PupA is expressed and pseudobactin 358 is efficiently transported in a PfrA-defective background. It is therefore likely that autoinduction of siderophore synthesis in *P. putida* WCS358 relies on a different combination of the response regulator and ECF sigma factor[91].

Autogenous regulation of pseudobactin-mediated iron uptake sytem was also observed in the plant-growth promoting strains of *Pseudomonas*

fluorescens M114 and *Pseudomonas* B10. In *P. fluorescens* M114, transcription of the pseudobactin M114 biosynthesis gene *pbsC* and of the cognate receptor gene *pbuA* is dramatically reduced in pseudobactin biosynthetic mutants compared to wild-type M114[25]. Addition of pseudobactin M114 to the growth medium markedly increases activity of both biosynthetic and receptor gene promoters. Both *pbsC* and *pbuA* are under the control of the IS ECF sigma factor PbrA[132]. Since *pbrA* expression was not reduced in the siderophore biosynthetic mutants, it was argued that pseudobactin M114-mediated induction of *pbsC* and *pbuA* does not take place through activation of *pbrA* transcription. A similar behaviour was reported for *Pseudomonas* B10. In this strain, the pseudobactin B10 biosynthetic gene *psbA* is under the control of the IS sigma factor PsbS, which in turn is controlled by Fur[3, 80]. A mutation in the *psbA* coding sequence dramatically reduces transcription of *psbA* without affecting the expression of PsbS sigma. However, addition of pseudobactin B10 to the *psbA* mutant fully restores transcription of *psbA*[4]. Taken together, these data indicate that endogenous pseudobactins, as the end-product of their biosynthetic pathways, are key participants in the control circuit regulating their own production. As reported in Section 4, the recent identification of a hybrid signal transduction pathway starting from pyoverdine binding to the cognate receptor made it possible to fill the gap between siderophore signalling and autogenous regulation of siderophore synthesis in *P. aeruginosa*.

4. A DIVERGENT SIGNAL TRANSDUCTION PATHWAY REGULATES PYOVERDINE SYNTHESIS AND UPTAKE

Pyoverdines (*syn.* pseudobactin in soil pseudomonads) are a heterogeneous class of fluorescent siderophores produced by the rRNA-homology group I pseudomonads and a few newly described species[93]. As a rule, all these siderophores are composed of three domains: (a) the fluorescent dihydroxyquinoline chromophore, which is structurally conserved in all pyoverdines; (b) an acyl side chain (either dicarboxylic acid or amide) bound to the amino group of the chromophore; (c) a variable peptide linked via amide bond to the C1 (rarely C3) carboxyl group of the chromophore. This peptidic domain is endowed with a great strain-dependent diversity that reflects uptake specificity. It contains both usual and unusual amino acids, the latter including acylated or cyclic forms of Nδ-hydroxyornithine, diaminobutyrate, β-hydroxyaspartate, β-hydroxyhistidine, and D-amino acids[93]. Fe(III) is bound by all pyoverdines with very high affinity ($K_f \approx 10^{32}\ M^{-1}$), leading to the formation of a hexadentate complex involving the hydroxyl residues of the chromophore as well as hydroxamate, hydroxyl, and/or carbonyl groups of the

peptidic moiety. Notably, the typical fluorescence emission of all iron-free pyoverdines is quenched upon iron binding, and this behaviour facilitated the investigation of some functional properties of these siderophores[48]. NMR and X-ray studies highlighted remarkable differences of the 3-D structure between different pyoverdines, except for the observation that iron is always coordinated at an edge of the structure, defining an open access side. This lack of structural similarity explains the strict recognition and transport specificity of different pyoverdines and corroborates the role of the peptide moiety in determining receptor specificity[48]. To date, more than 40 different pyoverdines were characterised and grouped in at least 3 different types, defined according to isoelectrofocusing and cross-feeding patterns, providing the rationale for a novel typing system of fluorescent pseudomonads based on pyoverdine heterogeneity[93]. The structure of pyoverdine from the type strain of *P. aeruginosa* PAO1 (ATCC 15692) is illustrated in Figure 3 (panel A).

Pyoverdine biogenesis is a complex and yet poorly understood process. Both the chromophore and the peptide moiety of pyoverdines are synthesised by non-ribosomal peptide synthases[31]. These large modular enzymes carry on a multistep reaction according to the carrier thiotemplate mechanism, implying that pyoverdine diversity can primarily be ascribed to the different organisation and substrate specificity of such enzymes[1, 77, 79, 98, 104]. A variety of different enzymes are involved in pyoverdine biosynthesis, and the corresponding (*pvd*) genes appear to be clustered in a single locus encompassing more than 120 kb of the *P. aeruginosa* chromosome[144] (http://www.pseudomonas.com), presumably including 37 open reading frames, as shown in Figure 3 (panel B).

After (or while) assembled, pyoverdine is exported into the extracellular milieu by a still uncharacterised, twin arginine translocation (TAT)-dependent secretion machinery. In fact, *tat* mutants of *P. aeruginosa* are unable to release pyoverdine and to use pyoverdine as iron source, indicating that not only pyoverdine, but also its receptor require the TAT translocon for secretion[104]. When excreted in the medium, pyoverdine delivers iron to the cell through receptor-mediated uptake. The iron–pyoverdine complex dissociates into the cell, but pyoverdine is not degraded as it is for most siderophores. Indeed, like aerobactin, pyoverdine is recycled and the same molecule performs several rounds of iron transport, saving precious energy otherwise required for its biogenesis[127].

4.1. The FpvA Receptor: Involvement in Pyoverdine Transport and Biosynthesis

The ferric pyoverdine receptor is an iron-regulated outer membrane protein of 86 kDa, encoded by the *fpvA* gene[115, 116]. FpvA shows the typical features of TonB-dependent receptors involved in surface signalling, like

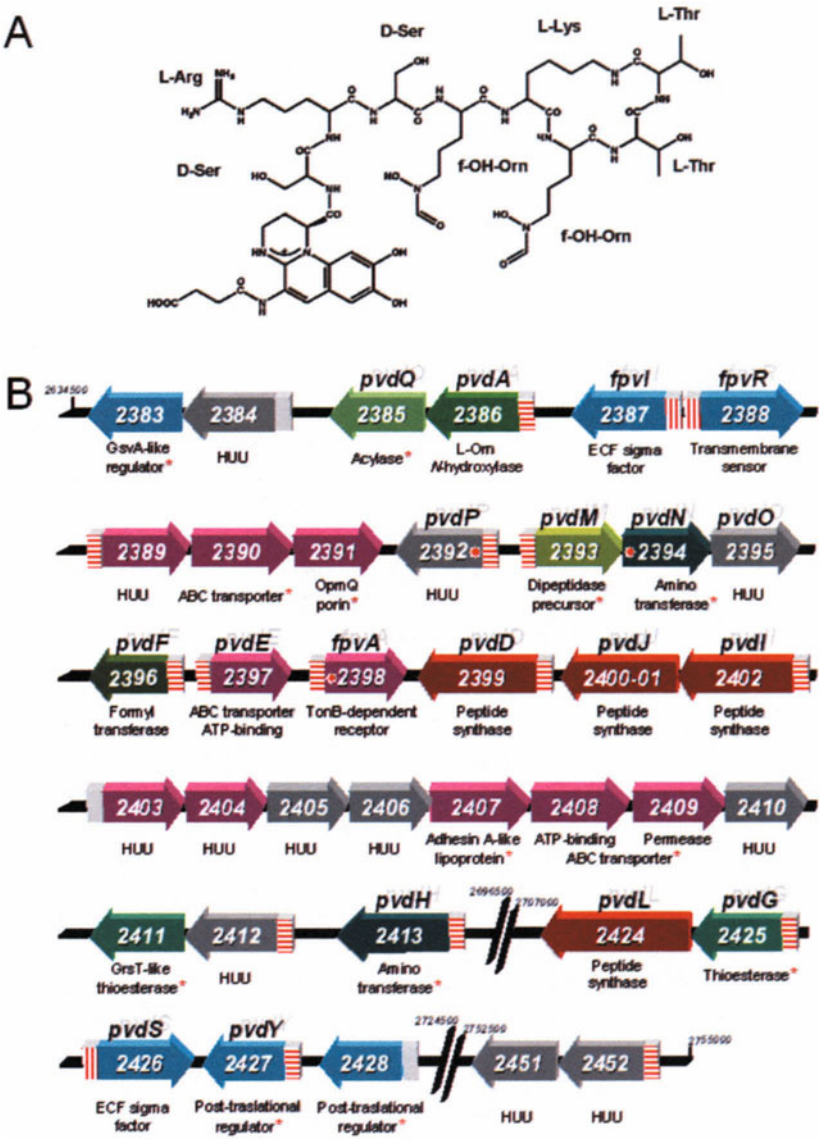

Figure 3. The pyoverdine system of *P. aeruginosa* PAO1. A. Chemical structure of pyoverdine. f OH-Orn is N^5-formyl-N^5-hydroxyornithine; the side chain of the chromophore can be either an amide or a dicarboxylic acid as shown. B. The pyoverdine gene system. The whole locus is predicted to span from PA2383 (position 2634500) to PA2452 (position 2755000) and contains two insertions of 10.5 kb (from PA2413 to PA2424) and 48.5 kb (from PA2428 to PA2451) for a total length of approximately 72 kb. The actual involvement of PA2383-84, PA2390-91, PA2403-11 and PA2451-52 in pyoverdine-dependent iron uptake is still questionable[77, 104]. Coding sequences

FecA, PupA, PupB, and PbuA, though it differs significantly from these proteins at the sequence level (<50% similarity). Circular dichroism spectral analysis showed that FpvA is composed of 57% β-structure, 3% α-helix, and 40% random coils, similar to porins and siderophore receptors[129]. According to a low-resolution model, FpvA is predicted to be composed of an N-terminal domain of 62 amino acids implicated in signal transduction (FecR box), followed by a putative TonB box (presumably ATMITS at position 129–134 of the unprocessed protein), a globular (plug) domain, and the C-terminal β-barrel spanning the outer membrane[48].

Transport of ferric pyoverdine through FpvA is an energy-dependent process and three TonB homologues (*tonB1*, *tonB2*, and PA0695) have been identified in the *P. aeruginosa* genome[113, 168, 169]. The *tonB1* gene is iron-regulated, Fur repressible, and essential for siderophore-mediated iron uptake[113]. However, TonB1 differs from the *E. coli* homologue for an extra sequence of *c*. 90 amino acids at the N-terminus and the absence of genetically linked *exbBD* genes. The N-terminal extension is essential for TonB1 activity and prevents TonB1 degradation, just like ExbB stabilises TonB in *E. coli*[169]. Homologues of *exbB* and *exbD* were identified downstream of the *tonB2* gene, but the whole *tonB2* gene system seems to be dispensable for iron acquisition[168]. It is therefore possible that TonB1 acts in concert with the *exb* homologues encoded by PA0693 and PA0694, or with other proteins, or that the N-terminal extra sequence renders TonB1 capable of working autonomously. Mutational analysis indicates that some residues are critical for TonB1 activity in both *E. coli* and *P. aeruginosa*, while other differentially affect TonB1 function. In particular, mutation of the conserved His98 residue within the membrane anchor sequence (corresponding to the protonable His20 in *E. coli* TonB, see Section 3.2) and of several residues in the conserved C-terminal domain abrogate TonB1 activity in *E. coli* but not in *P. aereuginosa*. Clearly, the TonB system of *P. aeruginosa* is endowed with structural and functional peculiarities compared with the homologous system of *E. coli*[169].

It has been assumed for a long time that siderophore receptors selectively recognise the ferrated forms of their ligands, because binding of the

Figure 3. Continued
(not to scale) are oriented according to the direction of transcription. Gene annotations and map positions refer to the Pseudomonas Genome Project (http://www.pseudomonas.com). Promoters are shown as boxes upstream of each transcriptional unit. Vertical red bars are Fur boxes; horizontal red bars are IS boxes. The TAT signals are represented by red stars. Red asterisks show protein functions inferred from *in silico* prediction. Gene products with unknown function are indicated by the HUU acronym (hypothetical, unclassified, unknown). Colours designate predicted or confirmed protein functions, as follows: blue, regulators; dark pink, membrane proteins; red, peptide synthases; green tonalities, biosynthetic enzymes belonging to other families; grey, HUU polypeptides. For further details and references, see the text.

apoform would be unproductive. This dogma was proven to be false in the case of FpvA, which is indeed capable of binding both unsaturated and iron-saturated pyoverdine, suggesting that pyoverdine-loaded FpvA can be the normal state of the receptor. Evidence for this unusual property originated from the observation that FpvA co-purifies with pyoverdine[129]. Fluorescence resonance energy transfer (FRET) was then used to study the dynamics of pyoverdine binding to FpvA both in vitro and in vivo. Pyoverdine was purified from wild-type (pyoverdine producing) *P. aeruginosa*; after excitation of the Trp residues of FpvA at 290 nm, emission peaks at 337 nm and 447 nm were observed, corresponding to the fluorescence of tryptophans and apo pyoverdine, respectively. Purification of FpvA from a pyoverdine-defective strain gave a single emission peak at 337 nm, but no evidence of fluorescence emission at 447 nm. This indicates that pyoverdine is normally located in the binding pocket of FpvA at a short distance from excitable tryptophans.

In vitro, the FpvA–pyoverdine complex shows very slow kinetics of saturation with iron, upon addition of either ferric (di)citrate or ferric pyoverdine. Nonetheless, FpvA binds in vitro both apo and iron-loaded pyoverdine with equally high affinity and 1:1 stoichiometry[129]. These findings raised the question of how, under the normal iron-limited conditions, few iron-loaded pyoverdine molecules can compete with a large excess of apo pyoverdine for productive FpvA binding. An answer to this question came from the recent demonstration that, in vivo, the affinity of ferric pyoverdine for FpvA is 17-fold higher than that of apo pyoverdine, though the two forms show comparable affinities for FpvA in vitro. Moreover, FpvA-bound apo-pyoverdine is not transported by *P. aeruginosa*, while the iron-loaded siderophore can efficiently enter the cell[128]. FRET studies also indicated that iron-saturated pyoverdine does not exchange the metal with the receptor-bound apo siderophore in vivo; rather, ferric pyoverdine quickly replaces FpvA-bound apo pyoverdine, showing fast displacement kinetics that parallel the rate of iron uptake. This mechanism is fully consistent with the uptake specificity of FpvA, that selectively recognises the homologous pyoverdine from strain PAO1. Exchange between siderophores would be incompatible with such receptor specificity. The TonB system (TonB1 and TonB2) appeared to be essential for the efficient displacement of FpvA-bound apo pyoverdine by ferric pyoverdine[128]. However, binding of deferrated pyoverdine was not influenced by TonB, suggesting that recognition of the ferrated siderophore requires a different, transport-competent geometry of FpvA. According to this model, the TonB system(s) could drive a conformational change that facilitates release of the apo siderophore and replacement by the ferrated form. Since only ferrated pyoverdine is taken up by the cell, while the apo siderophore is not, the possibility that FpvA adopts different conformations depending on its loading status was experimentally tested. Preliminary data indicate that the

FpvA–pyoverdine complex adopts two different structures depending on whether it is formed in vitro or in vivo[47].

The intriguing issue concerning the functional role of the N-terminal extension (the FecR box) of FpvA has recently been addressed[136]. It is apparently illogical to find such extension among receptors for endogenous siderophores (e.g., FpvA), that would be coordinately expressed together with the cognate siderophore (e.g., pyoverdine) biosynthetic genes. While this is generally true, in the case of FpvA and some other pyoverdine receptors (reviewed in ref. [114]) there is an additional level of functional complexity, being the same receptor involved in pyoverdine transport, regulation of pyoverdine synthesis and regulation of its own expression. Intriguingly, a Δ*fpvA* mutant was defective in both pyoverdine transport and synthesis, and showed reduced transcription of the PvdS-regulated gene *pvdD*, which encodes a pyoverdine synthase[1]. These defects were complemented by a chromosomal insertion of *fpvA*, but not by a truncated version of *fpvA* lacking the 5′ (N-terminal) coding region. Indeed, truncated FpvA did not restore pyoverdine synthesis, but it was able to transport the siderophore[136]. These observations are consistent with the modular structure predicted for FpvA, with the N-terminal extension involved in signalling and the rest of the protein involved in pyoverdine transport[48]. Moreover, the implication that FpvA controls the synthesis of the homologous siderophore has a wider biological sense. In those species, like *P. aeruginosa*, that produce two siderophores (i.e., pyoverdine and pyochelin) and utilise a variety of exogenous iron chelates, the coupling of siderophore uptake with siderophore biosynthesis could ensure that a certain siderophore is produced only under conditions in which it is effective in delivering iron to the cell, namely when it is productively engaged with the cognate receptor.

4.2. Divergent Regulation of Pyoverdine Biosynthesis and Transport: The PvdS and FpvI Sigma Factors

It is well known that pyoverdine synthesis and uptake genes lack Fur-dependent regulatory elements in their promoters and cannot be expressed in *E. coli*. On this basis it was assumed that one or more specific transcription factors, which are present in *P. aeruginosa* but not in *E coli*, are responsible for the recruitment of RNAP on pyoverdine promoters and for iron regulation of the pyoverdine gene system. This assumption was proven to be correct in 1995, thanks to the identification of the Fur-repressible gene *pvdS*, encoding an alternative sigma factor highly homologous to IS ECF sigmas[32, 95]. PvdS is a 187-residue protein that forms stable complexes with the core-RNAP from both *P. aeruginosa* and *E. coli*, and requires the presence of core-RNAP to bind the promoters of pyoverdine biosynthetic genes, including *pvdA, pvdE/F*,

and *pvdD*[81, 161]. PvdS was shown to cause RNAP core enzyme to initiate transcription from *pvdD* and *pvdE/F* promoters in vitro[161], consistent with its function as a sigma factor.

Like all ECF sigma factors, PvdS lacks most of the conserved region 1 that in RpoD prevents DNA binding in the absence of core RNAP. Nevertheless, PvdS is unable to associate with DNA in the absence of core RNAP[81, 161], suggesting that a different mechanism must exist to prevent promoter binding by free PvdS. Despite the reduced size of all ECF sigma factors compared with RpoD, the mechanism of promoter recognition is likely to be similar in RpoD and ECF sigma factors, including PvdS. There is a clear sequence similarity between PvdS and RpoD in both regions 2.4 and 4.2, which in RpoD recognise the −10 and −35 hexamers[81]. Accordingly, mutations in both these regions interfere with the ability of core RNAP–PvdS complexes to bind promoter DNA[154]. Moreover, the interactions of RpoD and PvdS with core RNAP are likely to be similar, with mutations in the conserved regions 2.1 and 2.2 of both sigmas reducing core-binding activity and comparable numbers of residues being involved[135, 154].

A typical signature of all PvdS-dependent promoters is represented by a conserved sequence known as the IS box, located approximately at position −33 relative to the transcription startpoint. Mutational analysis indicates that this sequence element is essential for promoter activity in vivo and in vitro[162], acting as the −35 motif of RpoD-dependent promoters. This sequence is also present in the promoters of genes regulated by the PvdS homologues PfrI, PbrA, and PsbS[125]. The PvdS-dependent promoters also have, at a distance of 16 or 17 nucleotides downstream of the IS box, the consensus sequence CGT that is required for promoter activity. Consequently, the PvdS-dependent promoters are characterised by the consensus TAAAT-$N_{16/17}$-CGT. This motif has been recognised, with minor variations, in the promoter region of 19 out of 26 PvdS-regulated genes identified so far[104].

PvdS is not the only alternative sigma factor of the pyoverdine gene system. Recently, two independent research groups provided compelling evidence for the involvement of an additional IS ECF sigma factor, FpvI, in the transcriptional control of the *fpvA* receptor gene[11, 121]. Though the function of FpvI has been established only recently, the *fpvI* gene, formerly named *pig32*[102] and *pvdY*[82] had previously been identified as an iron-controlled transcriptional unit. In our laboratory, the *pvdY* gene sequence has been characterised in the mid-1990s (EMBL accession no. Z25465), and its iron-dependent regulation documented[82]. More recently, the *fpvI* gene (PA2387) was retrieved by homology searches for IS ECF sigma factors in the PAO1 genome, and was found to be located in the *pvd* locus, between *pvdA* (PA2386) and the *fecR*-like gene *fpvR* (PA2388)[76]. Both *fpvI* and *fpvR* are iron regulated at the transcriptional level[102, 104] due to the presence of Fur-repressible,

divergent promoters likely recognised by RpoD-dependent RNAP. Remarkably, in vitro binding of Pa-Fur to the intergenic region between *fpvI* and *fpvR* has been experimentally confirmed[102]. A role of FpvI in the regulation of FpvA expression was inferred by the strong reduction of FpvA levels in Δ*fpvI* mutants. The low expression of FpvA was due to reduced transcription of *fpvA* in the Δ*fpvI* background[11, 121]. Nonetheless, a basal level of FpvA expression was observed in PAO1, irrespective of the presence of *fpvI* and of the iron concentration of the medium[121]. This can be related to the fact that, different from $P_{fecABCDE}$, the *fpvA* promoter apparently lacks a Fur box and can be more permissive to baseline transcription in the presence of iron.

Since FpvA is also implicated in the control of pyoverdine synthesis genes, it was not surprising that both pyoverdine production and transcription of *pvdD* were severely reduced in the Δ*fpvI* mutant[11], similarly to what was observed in the Δ*fpvA* background[136]. Moreover, the positive effect of pyoverdine on FpvA expression was abrogated in the Δ*fpvI* mutant, while overexpression of *fpvI* strongly increased FpvA levels, irrespective of the presence of pyoverdine[11, 121]. These data indicate that pyoverdine, through signalling via FpvA, is implicated in, but not essential for, FpvA expression. Such a role for pyoverdine in the positive regulation of both biosynthesis (*pvd*) and transport (*fpvA*) genes also explains why *fpvA* transcription was found to be strongly reduced in a Δ*pvdS* mutant that was obviously impaired in pyoverdine synthesis[104].

4.3. Hybrid Signal Transduction Pathway from FpvA to PvdS and FpvI: The Dual Role of FpvR

The data just presented pose the question of how the FpvA receptor can modulate the activity of the two sigma factors PvdS and FpvI. The answer was provided by the identification of the *fpvR* gene, encoding an antisigma sensor protein homologous to FecR and PupR[76]. As anticipated, the *fpvR* gene is in opposite orientation relative to *fpvI*, and is transcribed from a Fur-regulated promoter[11, 102, 121]. FpvR is a 331 amino acid protein with a predicted single transmembrane helix (residues 93–115) and a cytoplasmic N terminus. The overall FpvR topology is very similar to that observed for FecR and PupR, and the similarity extends to the functional level. There is a high conservation of Trp residues in the N-terminal region of sensor proteins implicated in siderophore signalling, including FpvR[91], and the corresponding residues of FecR are known to be critical for signal transduction to FecI[142].

FpvR has typical antisigma functions, and its overexpression causes a strong transcriptional repression of *pvd* and *fpvA* genes, halting pyoverdine production and uptake. Conversely, a deletion of *fpvR* has only a minor

influence on pyoverdine synthesis and transcription of *pvd* and *fpvA* genes. These effects are consistent with the duplicate specificity of FpvR, inhibiting the activity of both PvdS and FpvI. Indeed, FpvR has no direct effect on the transcription of *pvdS* and *fpvI*, though both these genes were upregulated in the FpvR-overproducing strain due to reduced iron intake[76].

FpvA is clearly involved in signal transduction to FpvR, as inferred by the observation that transcription of *fpvA* and *pvd* genes is strongly reduced in the *fpvA* mutant, while this effect is reversed in the *fpvAfpvR* double mutant[11, 76]. The N-terminal part of FpvA has been proposed to be located in the periplasm[76], and the presence of a membrane-spanning domain in FpvR suggests that this protein exposes the C-terminal region to the periplasm. Accordingly, a truncated version of FpvA lacking N-terminal region was able to transport ferric pyoverdine, but could not direct pyoverdine synthesis because it was unable to transmit the activation signal to FpvR[136]. By analogy with the Fec model of *E. coli*, the C-terminal (periplasmic) domain of FpvR is predicted to interact with the N-terminal extension (FecR box) of FpvA. In the presence of FpvA-bound ferric pyoverdine, this interaction becomes productive and a signal is transmitted to the cytoplasmic (N-terminal) portion of FpvR determining the activity of the two sigma factors PvdS and FpvI.

Despite the overall similarity between the Fec or Pup and the FpvA / FpvR / FpvI-PvdS systems, some relevant differences are evident: (a) the FpvA / FpvR / FpvI-PvdS system is a self-controlled device that responds to an endogenous siderophore rather than to an exogenous chelate, as for the Fec and Pup systems; (b) FpvR is an antisigma *sensu stricto*, that is, it can only inhibit the activity of both PvdS and FpvI, while FecR and PupR are both required for positive and negative control of FecI and PupI activity; (c) FpvR can interact with two partners, PvdS and FpvI, while FecR and PupR have a strict specificity for the corresponding ECF sigmas, FecI and PupI, respectively; (d) the *pvdS, fpvI*, and *fpvR* genes are transcribed from independent promoters, while *fecIR* and *pupIR* are dicistronic units, as most of FecIRA-like siderophore signalling systems[154]. This peculiarity could also account for an adequate expression of FpvR to fulfil its duplicate antisigma activity, rendering possible the contemporary interaction with both PvdS and FpvI. As to this point, it should be reminded that translational coupling of the *fecIR* message was suggested to facilitate the recognition between the C-proximal domain of the FecI and the N-terminal region of FecR, yielding a 1:1 sigma–antisigma complex[147]. This is not the case of the *P. aeruginosa* pyoverdine signalling system, that is characterised by an independent transcriptional organisation of *pvdS, fpvI*, and *fpvR* genes. Here, the lack of translational coupling could also make possible the duplicate interaction between the cytoplasmic domain of FpvR and either PvdS or FpvI.

It is also worth mentioning that, in addition to PvdS, FpvI, and FpvR, other regulators of the pyoverdine system have been identified. Expression of

the *pvc* genes, that were suggested to affect in some way the synthesis of the pyoverdine chromophore, is also controlled by the LysR-type regulator PtxR[143], which in turn is controlled by PvdS[149]. Intriguingly, the *ptxR* gene is absent in fluorescent pseudomonads other than *P. aeruginosa*[149] implying that regulation of expression of the *pvc* genes occurs differently in other species. It should also be mentioned that expression of pyoverdine synthesis genes in *P. putida* requires the presence of the PfrA protein, in addition to the PvdS homologue PfrI[150]. PfrA is similar to the AlgQ (or AlgR2) protein from *P. aeruginosa* which is likely to act as an antagonist of RpoD[41]. It can therefore be speculated that PfrA enables PfrI to compete with RpoD for RNAP core enzyme in *P. putida*. However, AlgQ is not essential for the expression of *pvd* genes in *P. aeruginosa*[81]. Thus, while the role of IS sigma factors in directing the expression of genes required for the synthesis of pyoverdines is conserved among fluorescent pseudomonads, other regulatory factors involved in modulation of siderophore gene expression differ between pathogenic *P. aeruginosa* and harmless *Pseudomonas* species. An integrated scheme for pyoverdine biosynthesis, transport, regulation, and signalling is shown in Figure 4.

5. AUTOGENOUS CONTROL OF THE PYOCHELIN SYSTEM: INVOLVEMENT OF AN ARAC/XYLS-LIKE REGULATOR

5.1. The Pyochelin Gene System

Pyochelin is a thiazolidine siderophore produced by *P. aeruginosa* and some *P. fluorescens* strains (reviewed in ref. [114]). Pyochelin binds iron with 2 : 1 stoichiometry and relatively low affinity, but it can form complexes with a variety of transition metals[153]. Biosynthesis of pyochelin requires two iron-regulated operons, *pchDCBA* and *pchEFGHI*[123, 130]. A regulatory gene, *pchR*, lies upstream of the *pchD*, while the pyochelin receptor gene *fptA* is located downstream of *pchI*[31]. The biochemistry of pyochelin biogenesis has been elucidated in great detail (for a review see ref. [31]), and it has been possible to reconstitute the entire pyochelin biosynthetic pathway in *E. coli*[122]. Synthesis starts with the conversion of chorismate to isochorismate and then to salicylate, dependent on the activity of PchA and PchB, respectively[130]. Salicylate is a good iron chelator and can be released in the medium acting as a promoter of iron uptake in *P. aeruginosa*[152]. It also represents the substrate of the adenylating enzyme PchD that activates salicylate by AMP transfer to its carboxyl group. PchC is a thioesterase and might account for the release of intermediates formed from activated salicylate. In fact, detectable amounts of the antibiotic dihydroaeruginoic acid, a by-product obtained from the condensation of salicylate with one cysteine molecule, are

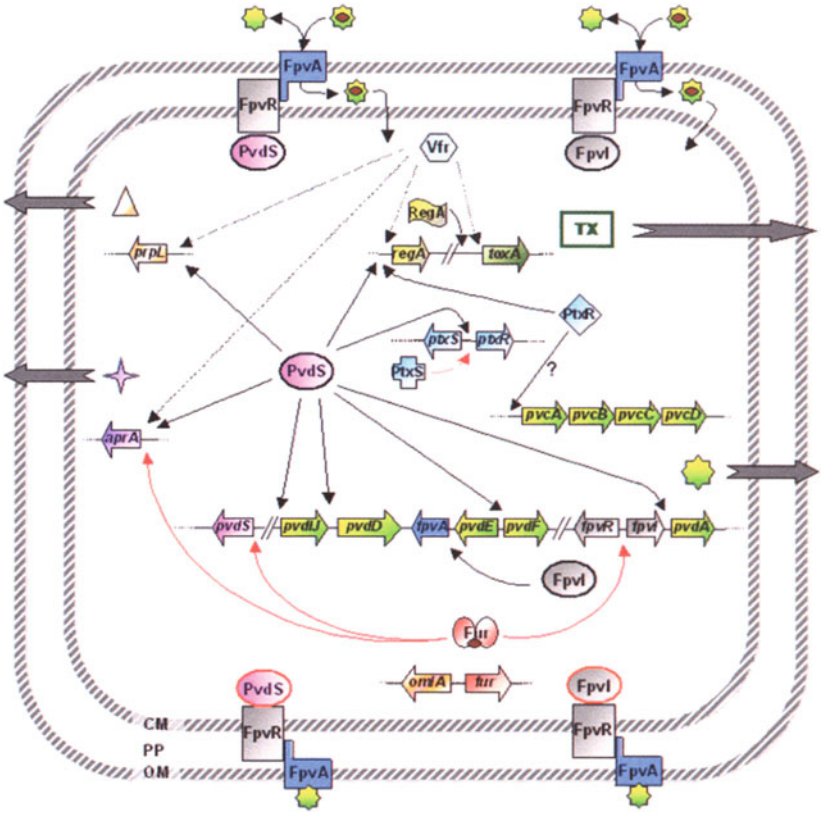

Figure 4. Model of iron-dependent regulation and pyoverdine signalling in *P. aeruginosa* PAO1. In the presence of sufficient intracellular iron, expression of pyoverdine and of some virulence factors is repressed by the binding of Pa-Fur-Fe(II) complexes to the Fur boxes located within the promoters of Fur-regulated genes (solid red arrows). When intracellular iron becomes limiting, the Fur repressor dissociates from target promoters, making possible the transcription of numerous genes, among which those encoding the FpvR sensor and the ECF sigma factors PvdS and FpvI. Ferric pyoverdine acts as the surface signalling molecule which indirectly regulates PvdS and FpvI activity. In the presence of iron-free pyoverdine, the activity of both PvdS and FpvI is suppressed by the inner membrane-spanning antisigma factor FpvR, as depicted in the lower part of the figure (inactive PvdS and FpvI are circled in red). Iron-loaded pyoverdine displaces the FpvA-bound apo siderophore and leads to a conformational transition of the FpvA which results in pyoverdine transport and release of PvdS and FpvI from the FpvR antisigma (top of the figure; active PvdS and FpvI are circled in black). Hence, PvdS and FpvI bind the core fraction of RNAP (not shown) to direct transcription of target genes (solid black arrows). FpvI is responsible for transcription of the *fpvA* receptor gene, while PvdS directs the expression of pyoverdine biosynthetic genes (*pvd*) and of virulence products, including the PrpL endoproteinase (*prpL*), the alkaline metalloproteinase (*aprA*), and the *toxA* regulatory genes *regA* and *ptxR* (TX is exotoxin A). Transcription of the *ptxR* gene is indirectly regulated by the GalR-LacI-like repressor PtxS, encoded by the *ptxS* gene (dashed red arrow). Expression of the *pvc* operon, presumably involved in the synthesis of the chromophore moiety of the pyoverdine, is under the transcriptional control of both PvdS and the LysR-like activator PtxR. The virulence factor regulator Vfr orchestrates the expression of

produced in iron-limited *P. aeruginosa* cultures[131]. PchE and PchF are the peptide synthases implicated in the condensation of the salicyl moiety with two cysteine molecules[111, 123]. PchG is an NADPH-dependent reductase implicated in reducing the thiazolinyl group of pyochelin, while PchH and PchI are ABC-proteins with predicted pyochelin export functions[31, 122].

5.2. Autoregulation of Pyochelin Synthesis and Uptake

Pyochelin is transported into the cell via the FptA receptor, an outer membrane protein of 75 kDa that shows some sequence similarity with hydroxamate siderophore receptors and differs from signal-transducing receptor due to lack of the typical N-terminal extension[7]. The whole pyochelin gene system is under the control of the Fur repressor due to the presence of putative or confirmed Fur-binding sites in the promoter regions of *pchR*, *fptA*, *pchDCBA*, and *pchEFGHI*[102, 123, 131]. Accordingly, transcription of the whole pyochelin gene system is silenced under high-iron conditions[7, 66, 123, 131]. The AraC/XylS-like regulator PchR plays a central role in the transcriptional control of pyochelin synthesis and transport genes, and is itself autoregulated[67]. PchR was initially recognised as an activator of the pyochelin system, as inferred by the fact that a *pchR* mutant was deficient in the production of both the FptA receptor and pyochelin itself[66]. Later, it was shown that also pyochelin-deficient strains express very low levels of FptA and that exogenously added pyochelin restored FptA synthesis, thus pointing to an involvement of pyochelin in the PchR-mediated control of FptA[66, 67]. Interestingly, it was noticed that FptA expression by the pyochelin-defective mutant was restored in a *pchR* knock-out, suggesting that PchR represses the expression of FptA in the absence of pyochelin, while it activates FptA expression in the presence of pyochelin. PchR also regulates its own expression, acting as a repressor on the *pchR* promoter in the absence of a functional pyochelin synthesis and uptake system[67]. Both *pchDCBA* and *pchEFGHI* biosynthetic operons appear to be under a similar control mechanism by PchR. Mutation in either biosynthetic operons strongly reduced transcription from both *pchD* and *pchE* promoters, while addition of pyochelin restored promoter activity. The two promoters are also silent in the *pchR*-defective background, though in this case repression cannot be reversed by pyochelin[123]. It appears therefore that a combination of pyochelin synthesis and transport functions is required for PchR-dependent activation of *pchR*, *fptA*, *pchDCBA*, and *pchEFGHI* promoters. The dual regulatory behaviours of PchR, that acts both as an activator of the pyochelin system (in the presence of pyochelin) and as a repressor (in its absence), is

Figure 4. Continued
extracellular proteins in response to cell density (dashed black arrows). The divergently transcribed *fur* and the *omlA* genes are shown since they have overlapping promoter regions. Genes are not to scale. OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane. For further details and references, see the text.

consistent with its being an AraC/XylS-like protein. AraC regulates the transcription of genes involved in the catabolism (*araBAD*) and transport (*araFGH*) of arabinose. AraC is composed of three domains: The N-terminal, arabinose-binding domain, also responsible for dimerization; a flexible linker; the C-terminal DNA-binding domain. Like PchR, AraC acts both positvely and negatively on *ara* genes. AraC binds to alternative sites on the *araBAD* promoter depending on the presence or absence of arabinose (the effector molecule), and this results in either activation or repression of transcription, respectively (reviewed in ref. [51]). Though molecular mechanisms for promoter recognition by PchR have not yet been elucidated, some preliminary evidence suggest that they could be mechanistically similar to those determined for AraC binding to P_{BAD}. The promoter elements of *pch* and *fptA* genes contain degenerated −10 and −35 hexamers, a common feature of promoters dependent on activation by AraC / XylR regulators[7, 51, 66, 123, 131]. Moreover, two partially conserved heptameric repeats, CGAGGAA and CGTGGAT, were found upstream of *fptA* and *pchR* coding sequences, suggesting that these sequences could function in PchR binding. Unfortunately, the difficulty in obtaining purified PchR has hampered the conclusive demonstration of the DNA-binding specificity of this regulator[67].

Another major issue that deserves further investigation is the mechanism that leads to PchR activation by pyochelin. It is clear that PchR activates *pchR* transcription only when pyochelin is present, the siderophore acting as a sort of effector. In the presence of pyochelin, the increased levels of PchR determine the upregulation of *pchR*, *fptA*, *pchDCBA*, and *pchEFGHI* thereby leading to an exponential amplification of all components of the system (i.e., regulatory, transport, and biosynthetic genes) (Figure 5, panel A). Yet, the mechanism by which PchR senses the presence of pyochelin (or ferric pyochelin) is a matter of speculation. One possibility is that ferric pyochelin, once transported into the cytoplasm, binds the N-terminal (effector) domain of PchR switching its pattern of promoter recognition to transcription-competence. However, the mechanism by which pyochelin is transported through the cytoplasmic membrane is still obscure. The possibility also exists that an interaction between ferric pyochelin and FptA could initiate a signal transduction cascade which leads to the release of an effector which binds PchR to provide activation. Alternatively, binding of ferric pyochelin to FptA could induce a conformational transition of the receptor that is transmitted to a yet unknown transmembrane protein directly responsible for PchR activation. In the latter two options, ferric pyochelin does not have to be transported inside the cell for transduction of the signal of successful iron chelation, as in the case of pyoverdine system. Again, there is recurrent logic for the regulation of iron transport systems in *P. aeruginosa*: It is convenient to capitalise on the synthesis of a particular siderophore if it is effective in feeding the cell with iron. This logic rigidly applies also to the regulation of those genes encoding receptor proteins for exogenous iron chelates.

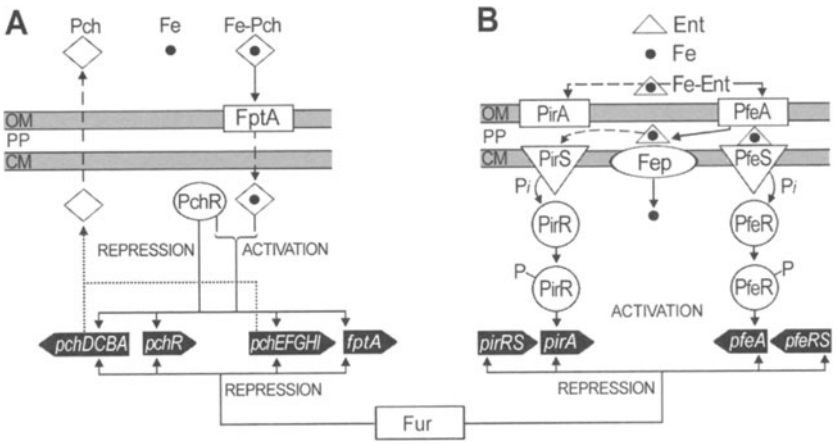

Figure 5. Autogenous regulation of the pyochelin and enterobactin gene systems in *P. aeruginosa*. A. Regulation of pyochelin synthesis and uptake. Pyochelin (Pch) is synthesised in the cytoplasm by the products of *pchDCBA* and *pchEFG* genes (dotted line). The *pchHI* genes encode for putative Pch export proteins. Ferric pyochelin (Fe-Pch) is transported through the FptA receptor. In the presence of Fe-Pch (right), the AraC/XylS-like regulator PchR behaves as an activator of transcription arising from *fptA*, *pchDCBA*, and *pchEFGHI*. In the absence of Fe-Pch (left), PchR acts as a repressor of *fpvA* and, presumably, *pch* genes. The whole *pch* gene system is under the control of the Fur repressor which blocks *pchR*, *fptA*, *pchDCBA*, and *pchEFGHI* promoters in the presence of sufficient intracellular iron. The transport route of Fe-Pch through the cytoplasmic membrane and the mechanism of PchR activation by Fe-Pch are still unclear processes. B. Regulation of enterobactin uptake. Enterobactin (Ent) chelates iron in the medium, and the ferric enterobactin (Fe-Ent) complex is translocated into the periplasm by the high-affinity receptor PfeA (right). Alternatively, Fe-Ent can reach the periplasm via a second (low-affinity) receptor, PirA (left). Both enterobactin transport systems, Pfe and Pir, are controlled by two-component regulatory systems. In the periplasm, Fe-Ent interacts with EnvZ-like histidine kinase sensors, PfeS or PirS, which phosporylate the cognate OmpR-like response regulators, PfeR or PirR, respectively. Induction of receptor genes does not require receptor triggering by Fe-Ent, but only a direct interaction of the sensor kinases components with Fe-Ent. Once phosphorylated, PfeR and PirR activate the transcription arising from *pfeA* and *pirA* promoters, respectively. Fe-Ent is then transported in the cytoplasm by the membrane-anchored Fep complex. Genes are not to scale. Dashed lines denote putative pathways. OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane. For further details and references, see the text.

6. SIGNALLING VIA EXOGENOUS IRON CHELATES

In Nature, the ability to utilise exogenous iron chelates is an important strategy for bacterial iron uptake. Ideally, the wider the ecological niche of a given bacterial species, the greater will be the variety of iron chelates encountered

in the environment. In complex ecosystems, many bacterial inhabitants compete for iron in the same niche. The outcome of inter- and intra-specific competition will depend on the efficiency of individual iron uptake strategies, including the ability to utilise a variety of heterologous siderophores and iron chelates.

P. aeruginosa is a magnificent example of iron-uptake capability, due to its ability to prey upon many exogenous siderophores. It can utilise iron complexed with a variety of exogenous chelators, including enterobactin, ferrioxamine, haem, haemoglobin, citrate, nitrilotriacetic acid, myo-inositol hexakiphosphate, and other compounds (reviewed in ref. [114]). While small iron chelates can enter the cell via a non-specific mechanism through the OprF porin[92], large complexes must be acquired through specific receptor-mediated transport systems. An increasing body of evidence indicates that such receptors are expressed in response to both iron deficiency and presence of the cognate iron chelate. This implies the existence of multiple regulatory devices capable of sensing the different iron chelates and signalling their presence to the cytoplasm for expression of cognate uptake genes. In this section, we will focus on those signalling system that have been, at least in part, characterised using biochemical and/or genetic approaches.

6.1. Enterobactin

Enterobactin (or enterochelin) is a powerful iron chelator synthesised by *Enterobacteriaceae* from the condensation of three molecules of *N*-(2, 3-dihydroxybenzoyl)-L-serine. *P. aeruginosa* does not synthesise enterobactin, but can use ferric enterobactin through receptor-mediated uptake[117]. Two enterobactin transport systems were identified in *P. aeruginosa* both of which are controlled by two-component regulatory systems (for a review on these systems, see ref. [124]). The high-affinity receptor for ferric enterobactin, PfeA, is an 80-kDa outer membrane protein highly similar to the homologous FepA receptor of *E. coli*[35]. Expression of PfeA is iron-repressible due to the presence of an experimentally confirmed Fur binding site in the *pfeA* promoter region[102]. The *pfeA* gene is preceded by two regulatory genes, designated *pfeR* and *pfeS*, encoding respectively an OmpR-like response regulator and a EnvZ-like histidine kinase sensor[36]. The *pfeR* and *pfeS* genes are cotranscribed from a single Fur-regulated promoter[34]. As a consequence, both *pfeA* and *pfeRS* are expressed in response to iron deficiency. The PfeR–PfeS regulatory pair possesses relevant structural features of two-component signal transducers, that is, the membrane-spanning (input) domain and the cytoplasmic (transmitter) domain which contains an autophosphorilable histidine residue in PfeS, and the two invariant aspartate residues acting as phosphate acceptors in the overall conserved PfeR sequence[34].

The *pfeA* gene is normally silent in iron-deficient *P. aeruginosa*, since PfeA expression requires the presence of (ferric) enterobactin in the periplasm[34]. Induction of *pfeA* transcription follows the binding of ferric enterobactin to the N-terminal periplasmic portion of the PfeS sensor kinase, triggering PfeS phosphorylation and phosphotransfer to the receiver domain of PfeR. Phosphorylated PfeR is a DNA-binding protein that activates transcription arising from the *pfeA* promoter. Coherently, a *pfeR* mutant is insensitive to enterobactin-dependent induction of *pfeA*, while overexpression of the *pfeRS* operon results in hyper-responsiveness to enterobactin induction of PfeA expression[34]. The failure of enterobactin to enhance *pfeRS* expression also indicates that PfeR is inactive on the *pfeR* promoter.

Different from the typical surface signalling systems, induction of PfeA expression does not require the initial binding of enterobactin to PfeA. This was inferred by the evidence that a *pfeA* mutant retained the ability of up-regulating *pfeA* expression in the presence of enterobactin, implying that enterobactin can be translocated across the outer membrane through a PfeA-independent mechanism. This is likely to occur via a second low-affinity transport system, encoded by the *pirA* and *pirRS* gene system[114, 148]. By this entry pathway, sufficient enterobactin could be transported into the periplasm to activate the PfeS–PfeR regulatory pair, and this would lead to amplification of the Pfe system and high-affinity uptake of enterobactin (Figure 5, panel B).

Having the possibility to synthesise two siderophores and prey upon a variety of exogenous iron carriers (see following sections), it is then logical that *P. aeruginosa* has established a rigid hierarchy for the expression of the different iron transport systems. A recurrent strategy is to take advantage of the presence of an exogenous iron carrier, saving on precious biosynthetic resources. However, usage of siderophores is also dependent on their iron chelating properties, and these can vary depending on the substrate from which iron must be withheld, as well as on a variety of physical and chemical factors. On this basis, it is not surprising that in iron-starved cells of *P. aeruginosa*, uptake of ferric-enterobactin suppresses pyoverdine-mediated and pyochelin-mediated iron-uptake; and in the absence of ferric enterobactin, pyoverdine-mediated iron-uptake supresses the pyochelin system[35]. This uptake hierarchy clearly reflects the affinity for iron of these three siderophores in a chemically defined medium, suggesting that the underlying strategy is to acquire the best available siderophore at a given time and place[114].

6.2. Haem

Haem is very abundant in animal tissues and represents a major source of iron for bacterial parasites. Lysis of the host cells, including haemolysis, is the first step in haem acquisition. Haem constitutes the prosthetic group of

numerous proteins and enzymes, from which it can either be extracted by high-affinity ligands called hemophores or released following chemical denaturation or proteolytic cleavage. *P. aeruginosa* produces at least four cytolytic factors, that is, phospholypase C, the glycolipid haemolysin, rhamnolipid, and exotoxin A[40, 101]. In concert with extracellular proteases, haem can be released from haemoproteins becoming available for receptor-mediated uptake. Bacterial parasites have therefore evolved multiple strategies to acquire haem or haemoglobin from the host, and the ability to use these compounds as iron sources is characteristic of a number of pathogenic species, including some *E. coli* pathovars, *Haemophilus influenzae*, *Neisseria* spp., *P. aeruginosa, S. marcescens*, *Shigella dysenteriae*, *Vibrio cholerae*, and *Yersinia* spp.[155]. Two experimentally confirmed and one putative haem uptake systems have been characterised in *P. aeruginosa*. The first system, designated *phu*, is composed of six adjacent genes: the *phuSTUVW* operon and the divergently transcribed *phuR* gene[101]. Both transcriptional units are under the control of Fur, and Pa-Fur binding to the intergenic region between the *phuS* and *phuR* has been documented[102]. The *phuR* gene encodes a TonB-dependent outer membrane receptor for haemin and haemoglobin, while *phuS* to *phuW* genes encode individual components of a typical ABC transporter system, namely, a periplasmic binding protein (PhuS) and the permease-ATPase system (PhuTUV). No clear function has been assigned to PhuW. As expected, mutations in either *phuR* or *phuSTUVW* markedly reduced haemin or haemoglobin utilisation, though residual capacity to grow on these compound was observed. This behaviour can be ascribed to the presence of additional, functionally unrelated haem uptake system, *has*.

The *has* system is based on the activity of a 20-kDa protein, the HasA hemophore, that catches haem with high affinity (in the order of 10^{-8} M) and brings it back to the HasR receptor, in a fashion that reminds us of siderophore-dependent iron uptake. HasA was identified in iron-poor culture supernatants of *P. aeruginosa* by the use of antibodies that recognised the homologous protein from *S. marcescens*[83]. HasA is synthesised as a precursor peptide with a C-terminal export signal, and is likely to be secreted by the type I secretion system of the ABC family[101] encoded by the putative *hasDEF* operon that maps downstream of *hasA*. HasA-neutralising antibodies strongly inhibit haemoglobin utilisation, suggesting that HasA is the high-affinity transporter of haemoglobin-bound haem in *P. aeruginosa*[83]. HasA-bound haem is transported via HasR, a TonB-dependent receptor containing a putative N-terminal signal transducing domain[146, 154]. The *hasR* gene maps upstream of *hasA* and is preceded by a Fur-repressible promoter that is likely to generate a dicistronic transcript with *hasA*. Deletion of *hasR* resulted in a dramatic reduction of haem or haemoglobin utilisation, indicating that *phu* is inefficient in providing the cell with haem in the presence of extracellular hemophore. Once inside the

cell, haem-bound iron is released by the activity of the *pig4* (or *hemO*) gene product[102, 104], an oxygenase implicated in oxidative degradation of haem[120].

Interestingly, the *hasR* gene (PA3408) is preceded by a FecIR-like sigma-sensor pair, *hasIS* (PA3410, PA3409), which is iron regulated and contains an experimentally confirmed Fur box in the putative promoter region[102, 104, 154]. The role of these two *P. aeruginosa* genes in the regulation of the *has* system has not yet been confirmed, though the regulatory model implying signal transduction from HasR, through HasS, to HasI is supported by the presence of a putative signal-transducing extension (FecR box) in the N-terminal region of the HasR receptor and the genetic linkage between *hasR* and the *hasIS* ECF sigma-sensor pair[154]. A similar organisation of regulatory genes has recently been reported for the *has* system of *S. marcescens*[126]. In this organism, binding of the haem-loaded HasA (but not its apo-form) to the HasR receptor initiates the signal transduction cascade via HasS to HasI, definitely showing that the inducer (haem-loaded haemophore) and the transported substrate (haem) are different molecules.

6.3. Miscellaneous Siderophore Signalling Systems and Evolutionary Considerations

Homology-based genome-wide searches for surface signalling systems in the *P. aeruginosa* chromosome[144] made it possible to retrieve a total of 11 putative or confirmed surface signalling systems, all of which encode individual components characterised by significant similarity to the FecIRA complex of *E. coli*[154]. These include the *fpvA/fpvR/fpvI-pvdS* and *hasISR* gene systems, plus nine additional genetic clusters which are very likely to encode surface signalling systems (Table 1). For all of them, iron regulation via Pa-Fur has been either documented[102] or deduced from sequence analysis of putative promoter regions[154]. Still, indirect or preliminary experimental evidence suggests their involvement in receptor signalling through the recognition of exogenous iron chelates[154]. For instance, uptake of the hydroxamate siderophore ferrioxamine occurs via FiuA (PA0470), a protein carrying the N-terminal extension typical of signal transducing receptors (Figure 6). FiuA is under the transcriptional control of a regulatory system encoded by the *fiuI* and *fiuR* genes (PA0472 and PA0471, respectively), which have previously been shown to undergo direct regulation by Pa-Fur[102]. Ferrioxamine uptake thus requires both iron deficiency and the presence of this siderophore, and has been proposed to take place through surface signalling from FiuA, via FiuR, to the IS ECF sigma factor FiuI[148]. Evidence for the involvement of citrate in iron transport has also been reported for *P. aeruginosa*[27, 61]. Ferric (di)citrate uptake by *P. aeruginosa* is predicted to be mediated by the FecIRA-like system (PA3899-3900-3901, respectively) that shows substantial sequence similarity with the *E. coli* counterpart. In fact, the

Table 1. Putative and confirmed siderophore signalling systems controlled by IS ECF sigma factors in *P. aeruginosa* PAO1[a].

Receptor	Antisigma-sensor	IS ECF sigma(s)	Iron regulation (Pa-Fur binding)[b]	Uptake specificity[c]	Genetic organisation[d]
FpvA PA2398	FpvR PA2388	FpvI (PvdS) PA2387 (PA2426)	Yes (confirmed)	Pyoverdine	←I R →/—/ A →/—/ I→
HasR PA3408	HasS PA3409	HasI PA3410	Yes (confirmed)	Haem	I→ R→ A→
HxuC PA1302	PA1301	PA1300	Yes (predicted)	Haem, putative	I→ R→ A→
FiuA PA0470	FiuR PA0471	FiuI PA0472	Yes (confirmed)	Ferrioxamine	I→ R→ A→
FecA PA3901	FecR PA3900	FecI PA3899	Yes (predicted)	Ferric citrate, putative	I→ R→ A→
OptS PA2466	PA2467	PA2468	Yes (predicted)	Undefined (ferrioxamine)	I→ R→ A→
OptI PA4897	PA4895	PA4896	Yes (predicted)	Undefined (haem)	←AI → R →
PA0151	PA0150	PA0149	Not observed (predicted)	Undefined (heterologous pyoverdine)	I→ R→ A→
PigC PA0674	PigE PA0676	PigD PA0675	Not observed (confirmed)	Undefined (heterologous pyoverdine)	A→ I→ R→
PA1365	PA1364	PA1363	Not observed (predicted)	Undefined (aerobactin)	I→ R→ A→
UfrA PA1910	PA1911	PA1912	Not observed (predicted)	Undefined (ferrichrome)	I→ R→ A→

[a]Gene names and identification numbers are according to the *Pseudomonas* Genome Project database (http://www.pseudomonas.com).
[b]Iron regulation stands for >3-fold increase of gene expression under low-iron conditions. Pa-Fur binding is predicted on the basis of DNA sequence similarity with the Fur box, and confirmed by Pa-Fur-DNA binding assays. Data are from refs [102, 104, 148, 154].
[c]When undefined, the receptor uptake specificity is predicted from that of the closest homologue (as retrieved from the site http://www.pseudomonas.com) and given in parenthesis.
[d]The arrows show the orientation of FecI (I), FecR (R), and FecA (A) homologues in the *P. aeruginosa* genome; the /—/ symbol denotes an intergenic region.

Figure 6. Sequence alignment of the signalling domain of *P. aeruginosa* FecA-like receptors. The N-terminal region (FecR box) of the putative FecA-like receptor proteins of *P. aeruginosa* was aligned with the signalling domain FecA and PupB. The numbering is relative to the start codon. Outer membrane targeting sequences were removed. The TonB box is not included. Identical residues are highlighted in reverse type; similar residues are shaded. The following sets of residues are considered similar: IVLM, RKHDE, PA, ST, NQ, FYW. The uptake specificity is experimentally confirmed for FecA, PupB, FiuA (PA0470), and HasR (PA3408) receptor proteins. For further details, see also Table 1.

P. aeruginosa Fec system is also likely to be Fur-regulated due to the presence of a Fur box upstream of the *fecIR* coding sequence, thus showing similarly arranged regulatory and transport genes[114, 154]. Additional surface signalling systems include a third, putative haem uptake system, preliminarily designated *hxu* (PA1300-PA1301-PA1302), and six more systems implicated in the uptake of still uncharacterised, presumably hydroxamate-like, siderophores. This wide uptake specificity would be consistent with the ability of *P. aeruginosa* to utilise aerobactin, various hydroxamate siderophores of fungal origin and heterologous pyoverdines[114]. At the genetic level, most of these putative surface signalling systems show the canonical arrangement, with the ECF sigma (*fecI* like) gene preceding the sensor (*fecR*-like) gene and the signal-transducing receptor (*fecA*-like) gene. The *pigCED* and the *optI*-PA4895-PA4896 gene systems are the only exceptions, in so far as the receptor gene precedes the sigma–antisigma gene pair (Table 1). At present, there is insufficient information on these systems to say if such an unusual arrangement also reflects on the functional level, as it does for the *fpvA/fpvR/fpvI-pvdS* system. Indeed, the uptake specificity of these putative surface signalling systems has still to be addressed, and in some cases their regulation by iron and Pa-Fur needs to be confirmed. In this context, it is interesting to point out that some of these systems escaped identification by DNA microarray analysis of *P. aeruginosa* iron-regulated genes (see the last four systems in Table 1), suggesting that induction of such systems requires the presence of an exogenous stimulus (e.g., an iron carrier) in addition to an iron-poor environment[104]. This is certainly true for the *pigCED* system, that has been proven to be directly controlled by Pa-Fur[102].

Though the majority of the *fecIRA*-like clusters retrieved from the *P. aeruginosa* genome share the canonical structure, 8 of the 18 *P. aeruginosa*

fecA-like genes are not associated with *fecIR*-like clusters, and three *fecIR* pairs (PA2050-51, PA2093-94, PA2387-88) are unlinked to a *fecA*-like gene (Table 1; see also ref. [154]). This could indicate that surface signalling is limited to those receptors which are genetically coupled with a *fecIR*-like unit, and/or that the same FecR-like signal transducing device can interact with more than one FecA-like receptor, enabling functional coupling of *fecIR*-like units with genetically unlinked *fecA*-like partners. Based on both evolutionary and functional considerations, both hypotheses are mutually compatible. Since the Fur protein and its recognition sequences are well conserved among diverse species, the Fur-controlled surface signalling systems may have ancestrally been transferred from a species to another without loss of master regulation[109]. Then, paralogous gene clusters arose during the evolutionary history of the species through sequential duplications of the ancestral (either *fecIRA*-like or *fecIR*-like) cluster, followed by functional and structural specialisation of gene copies. Divergence from the ancestor and acquisition of novel functional specificities is intuitively obvious for duplicated *fecIRA*-like clusters. However, the acquisition of a novel function by a duplicated *fecIR*-like cluster would require a pre-existing heterogeneity of FecA-like receptor proteins capable of interacting directly with the antisigma product of the duplicated *fecIR*-like cluster. This would enable genetically unlinked partners to evolve towards novel functional specificities. In both cases, duplication would be stabilised in the genome provided that functional complementarity between interacting partners (in the order, the FecA-, FecR-, and FecI-like proteins) and selective advantage due to acquisition of a novel function (e.g., autogenous regulation of receptor gene expression) are both ensured. In the course of divergence, the need of functional complementarity results in coevolution of interacting partners, a feature which is often highlighted by the topological similarity of gene families trees of cognate paralogs[50]. This evolutionary rule is unequivocal in the case of *P. aeruginosa fecIR*-like gene pairs. In fact, analysis of homology-based trees for IS ECF sigmas (www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG1595) and FecR-like antisigmas (www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG3712) provides compelling evidence of coevolution for functionally and genetically linked members of these two gene families[154]. Coevolution of FpvR with FpvI has also been documented[154], suggesting that FpvI could represent the evolutionary linked partner of FpvR. Similar conclusion were also inferred by the analysis of ECF sigmas-sensor pairs in *P. putida* KT2440[91]. In *P. aeruginosa*, only *pigCED* and *pvdS* genes escape this rule and, remarkably, they show an unusual genetic organisation. Thus, a canonical structure of the gene cluster is strongly suggestive of coevolution of the corresponding protein products.

The homology-based tree retrieved from full-length analysis of all members of the iron transport outer membrane protein family shows that FecA-like receptors from *P. aeruginosa* are scattered in several distinct branches which do

not correlate with the evolutionary pattern of genetically linked FecIR pairs (www.ncbi.nlm.nih.gov/cgi-bin/COG/palox?COG1629). We focussed on the N-terminal extension (FecR box) of signal transducing receptors of *P. aeruginosa*. Based on the FecA model, this region is predicted to be flexible (not crystallisable) and free to interact with FecR in the periplasm[46]. From a comparative analysis of putative FecR boxes of *P. aeruginosa* receptors, it appears that this functional domain is conserved in length and shows some sequence similarity in the border regions (Figure 6). This could indicate that a basic architecture is required for recognition of cognate sensor proteins by signal transducing receptors, and that the specificity determinants for sensor recognition reside in the variable regions of the FecR box. Despite the presence of conserved sequence elements in the putative signal-transducing domain of *P. aeruginosa* FecA-like receptors, attempts to infer a robustly supported tree from homology-based analysis of this domain have so far failed[154].

7. SIDEROPHORE SYNTHESIS, RECEPTOR SIGNALLING, AND THE REGULATION OF VIRULENCE GENES

7.1. Siderophore Production, In vivo Iron Uptake and Virulence of *P. aeruginosa*

Pyoverdine and pyochelin, the two siderophores produced by *P. aeruginosa*, are well known for being capable of scavenging Fe(III) from the host's iron-binding proteins in vitro, and have been associated with the pathogenicity of *P. aeruginosa* in different models of experimental infection. Dialysis equilibrium experiments suggested that both pyoverdine and pyochelin are able to cause the release of iron from ferric transferrin[140]. Pyoverdine can also remove iron from ferric lactoferrin in vitro, eventually assisted by *P. aeruginosa* elastase[163, 166]. The ability of both siderophores to withhold transferrin-bound iron results in growth promotion of *P. aeruginosa* in human serum or in laboratory media supplemented with human transferrin[8].

The importance of pyoverdine in the virulence of *P. aeruginosa* has been supported by direct evidences, including experimental infection in various animal models. Pyoverdine is known to be produced in vivo, during lung colonization in CF patients[54]. Pyoverdine-deficient mutants are less virulent than wild-type *P. aeruginosa* when injected into burned mice, and virulence can be restored by administration of purified pyoverdine during the infection[94]. Using a rat lung model of infection, that more closely mimics the respiratory infection of CF patients, colonization by *P. aeruginosa* was dependent on the

production of pyoverdine. Thus, pyoverdine is important for iron acquisition and growth in the lung environment, and could play a pathogenetic role in chronic pulmonary disease of CF patients[113]. Pyochelin also was shown to promote bacterial growth and lethality when co-injected with a virulent variant of *P. aeruginosa* PAO1 into the peritoneal cavity of immunocompromised mice[28]. Ferripyochelin is also responsible for secondary pathogenetic effects that derive from its ability to generate hydroxyl radicals from superoxide and hydrogen peroxide produced by neutrophils in response to the infection. It is also established that ferric pyochelin, alone or in combination with the phenazine pigment pyocyanin, contributes to the damage of pulmonary epithelial cells by catalysing the formation of cytotoxic hydroxyl radicals[21, 22, 38].

More recent studies based on the infection of immunosuppressed mice with genetically characterised siderophore-deficient mutants confirmed that pyoverdine and, to a lesser extent, pyochelin contribute to in vivo growth and virulence[145]. The Δ*pvdA* mutation, which blocks pyoverdine biosynthesis at a very initial stage, reduced *P. aeruginosa* growth in the mouse lung and prevents the septicaemic spread of infection. Comparatively, a Δ*pchD* mutant, defective in pyochelin and salicylate synthesis, was more invasive and virulent. In addition, the Δ*pvdA* Δ*pchD* double mutant was severely attenuated compared with single mutants, though it was still capable of surviving in vivo by virtue of its ability to acquire haem-bound iron[145]. In fact, neither growth in the lung nor dissemination of the infection were totally abrogated in all these mutants. On the contrary, a *tonB1* mutant that retained the ability to produce both siderophores but was impaired in TonB-dependent siderophore transport, could not grow in the immunosuppressed mice and showed no lethal effect[146]. Thus, both pyoverdine and pyochelin seem to be required, but not essential, for efficient in vivo multiplication of *P. aeruginosa* PAO1 and for virulence.

In line with the above observations, expression of siderophore biosynthesis or uptake genes has been documented in vivo. The *pvdI* gene, encoding a putative pyoverdine synthase, was detected among *P. aeruginosa* PAO1 genes expressed in vivo from both septicaemia and chronic lung infection models. The independent detection of *pvdI* from quite different types of animal infection could reflect the importance of pyoverdine-dependent iron acquisition in both mucosal and systemic disease[58]. Moreover, large-scale search for candidate virulence genes of *P. aeruginosa* strain PAK led to the identification of *fptA* (the pyochelin receptor gene) among those genes that are specifically induced in the respiratory mucus of CF patients[156]. The *fptA* gene requires both iron deficiency and the presence of pyochelin for transcription, suggesting that *P. aeruginosa* senses the CF mucus as an iron-poor environment and responds to iron deficiency with expression of the pyochelin gene system. Noticeably, *fptA* was also isolated from an additional screening of genes selectively expressed in a mouse model of infection, corroborating the importance of pyochelin in iron withholding from animal host tissues[157].

While siderophore-dependent iron transport systems certainly play a critical role in determining the infectious ability and virulence of *P. aeruginosa*, they can also represent a sort of Achilles' heel for this opportunistic pathogen. Given that siderophore systems ought to be expressed during in vivo growth, the potential of using siderophore-antibiotic conjugates as vehicles to deliver antibiotics into the bacterial cell has been exploited. In fact, human isolates of *P. aeruginosa* are becoming increasingly resistant to most antibiotics available for clinical use, and the reduced permeability of the bacterial outer membrane is often at the basis of resistance[57]. However, if antibiotics are taken up by active transport systems, as in the case of siderophores, it would theoretically be possible to bypass the outer membrane permeability barrier[16]. Linking of antibiotics to compatible sites of the siderophore molecule would result in high-affinity uptake, at least under conditions in which siderophore receptors are expressed. Receptor-mediated uptake would then increase (by a factor $\approx 10^3$) the intracellular concentration of antibiotic and lower its minimal inhibitory concentration. This promising approach, referred to as "Trojan horse strategy," is being pioneered by several investigators to increase the efficacy of anti-*Pseudomonas* drugs, including β-lactams and quinolones[23, 68, 72, 97] (and references therein).

7.2. Pyoverdine Signalling and the Regulation of Virulence Genes

As described earlier (Sections 4.2 and 4.3), binding of ferric pyoverdine to FpvA transmits a signal to FpvR which results in activation of both FpvI and PvdS sigma factors. Though the dichotomic pathway of signal transduction needs to be refined at the molecular level, there is compelling evidence that FpvA signalling affects the expression of several PvdS-regulated genes. Remarkably, while the specificity of FpvI seems to be restricted to the *fpvA* promoter[11, 121], PvdS is known to control a very large subset of genes within the Pa-Fur regulon, as shown in Figure 4[76, 104, 148]. At least three relevant virulence factors, namely exotoxin A, alkaline protease (AprA), and a type-IV (lysil class) endoproteinase (PrpL) have been shown to be under the direct transcriptional control of PvdS[100, 137, 160] and their expression could consequently be regulated via FpvA signalling[76].

P. aeruginosa produces several proteases that are implicated in tissue injury and host damage during infection. These enzymes hydrolyse tissue proteins providing the organism with essential amino acids, destroy connective fibers favouring bacterial spread from the primary site of infection, and alter the activity of the immune system acting on immunoglobulins and pro-inflammatory cytokines[86, 110]. The ability of *P. aeruginosa* proteases to cleave transferrin in the serum and neutrophil lactoferrin on the mucosal

surfaces also results in iron release and scavenging via siderophores[163]. *P. aeruginosa* proteases degrade human transferrins in the lung and create conditions that favour hydroxyl radical formation, ultimately contributing to severe lung injury in CF and chronic lung diseases[19]. These and other observations indicate that *P. aeruginosa* proteases could act cooperatively to increase iron availability and cause tissue damage[20].

Two proteases, PrpL and AprA, are preferentially produced in low-iron media. PrpL cleaves casein, lactoferrin, transferrin, elastin, and decorin, and enhances *P. aeruginosa* survival in a rat model of lung infection[160]. The AprA metalloprotease has been shown to hydrolyse transferrin thereby promoting the acquisition of transferrin-bound iron[137]. Analysis of *prpL* and *aprA* transcription indicates that both genes are up-regulated in response to IS[137, 160], and both *prpL* and *aprA* were recognised as iron- and PvdS-regulated genes using the *P. aeruginosa* DNA array[104]. Moreover, *prpL* expression positively responds to high pO_2 tension during the exponential growth phase, reminescent of *toxA* regulation[102]. Production of both PrpL and AprA was reduced, but not completely abrogated, in a Δ*pvdS* background[137, 160]. Complementation with the multicopy *pvdS* gene resulted in overproduction of PrpL by the Δ*pvdS* mutant[160]. A direct involvement of PvdS in *prpL* transcription was also inferred by the identification of a well-conserved PvdS-binding motif (TAAAa-N_{16}-CGT) in the *prpL* promoter. In contrast, no obvious PvdS-recognition sequences could be identified upstream of the *aprA* transcription start site. The *aprA* promoter shows the unusual feature of containing a functional though heavily degenerated Fur box[137], suggesting that Pa-Fur controls *aprA* expression both directly and indirectly via PvdS.

The hypothesis that pyoverdine is required for the expression of exotoxin A and PrpL protease was tested by the analysis of *toxA*::*lacZ* and *prpL*::*lacZ* transcriptional fusions in pyoverdine deficient and proficient backgrounds, and by direct measurements of exotoxin A production[76]. Transcription of *toxA* and *prpL* was significantly reduced in a *pvdF* biosynthetic mutant, while addition of pyoverdine restored transcription of both genes to wild-type levels. This effect paralleled the production profile of exotoxin A, inasmuch as the great reduction of toxin synthesis by the *pvdF* mutant was fully reversed by exogenously added pyoverdine. The regulatory role of pyoverdine was clearly mediated by the FpvA–FpvR–PvdS signalling cascade, since mutation of *fpvA* reduced exotoxin A production and abrogated pyoverdine-dependent induction. Thus, FpvA mediates the transmission of the pyoverdine signal to FpvR, and this enables PvdS to become transcriptionally active on target promoters, including those implicated in the expression of virulence factors like exotoxin A and protease(s). In the absence of signalling, however, FpvR behaves as a typical antisigma for PvdS, preventing the expression of virulence genes within the PvdS regulon. In line with this hypothesis, FpvR overexpression in

wild-type *P. aeruginosa* reduced the synthesis of pyoverdine and exotoxin A. Also in this case, repression could be relieved by an excess of exogenously added pyoverdine[76].

The importance of pyoverdine signalling for in vivo regulation of virulence gene expression remains to be elucidated. Noticeably, pyoverdines and their putative receptors appear to be expressed by in vivo growing *P. aeruginosa* cells[54, 58, 133, 134], and it is tempting to speculate that in biofilms or in microcolonies, which are the predominant modes of growth of *P. aeruginosa* both in vivo and in nature, the high bacterial density would favour siderophore-dependent signalling and cross-talk between cells. The little data available indicate that the FpvA–FpvR–PvdS system is activated by micromolar concentrations of exogenous pyoverdine[76]. In vitro, 10 μM apo pyoverdine is required for optimal induction, while the amount of free (i.e., receptor unbound) pyoverdine in pulmonary secretions of CF patients is approximately 10-fold lower[54]. However, these numerical estimations do not consider that the induction process is initiated by displacement of FpvA-bound apo pyoverdine by iron-loaded pyoverdine, which is indeed the prevalent form in CF sputa[54]. Therefore, an effective role of pyoverdine signalling in the CF lung secretion can be envisaged.

Recent data also indicate that PvdS expression is required for optimal virulence of *P. aeruginosa* in a rabbit model of experimental endocarditis[167]. It was reported that successful hematogenous dissemination of *P. aeruginosa* from an infected aortic (left) valve was dependent on the presence of a functional *pvdS* gene, while no bacterial spread from the tricuspid (right) valve was observed, irrespective of *pvdS*. This pattern of dissemination was correlated with the higher O_2 tension in the aerobic aortic side compared with the microaerobic tricuspid side (90 vs 40 mmHg, respectively) and, in turn, with the drastically reduced expression of *pvdS* under microaerobic conditions[100]. Moreover, the avirulent behaviour of the PvdS-defective strain was not dependent on reduced exotoxin A synthesis, suggesting that additional PvdS- and O_2-controlled factors, like pyoverdine and proteases, could facilitate dissemination from aortic vegetations. Related to these observations, it has been reported that biofilm formation by *P. aeruginosa* is inhibited by apo-lactoferrin (and other chelators) but not by ferric lactoferrin. By chelating iron, lactoferrin (and perhaps other chelators) stimulates surface motility of *P. aeruginosa* cells and prevents cell-to-cell aggregation and biofilm formation[138]. Given that the exposure to chelating agents, such as transferrins, upregulates PvdS expression and causes massive pyoverdine production by *P. aeruginosa*[29, 32, 137, 166], the enhanced invasiveness of PvdS-expressing cells could also be interpreted as a motorial response to low-iron and siderophore sensing. Although the relation of iron regulation and pyoverdine signalling to cell motility is unclear at the moment, there is growing evidence that the quorum sensing regulatory circuitry could provide an overlap

between these apparently unrelated aspects of *P. aeruginosa* physiology. In fact, synthesis of pyoverdine is affected by quorum sensing systems[159] and an integration of quorum sensing with iron homeostasis has been documented in *P. aeruginosa*, both phenomena primarily responding to high cell densities and nutrient exhaustion[14]. The understanding of the molecular mechanisms responsible for the cross-talk between quorum sensing, IS response, and siderophore signalling will be a future challenge.

8. CONCLUDING REMARKS

During the last decade it has become clear that positive control of siderophore transport is the rule in *P. aeruginosa*, and that siderophores account for at least three types of regulation and signalling in this bacterium. First of all, iron supply via siderophores enables *P. aeruginosa* to switch on the activity of Pa-Fur repressor thereby silencing the Pa-Fur regulon. Second, receptor binding by both endogenous and exogenous siderophores communicates the real efficacy of a given siderophore in iron delivery, acting as a stimulus for coordinate expression of the cognate uptake (and eventually biosynthesis) genes. Third, and apparently unique to the pyoverdine system, binding of the siderophore to the FpvA receptor activates a signal transduction cascade that leads to the overexpression of virulence-associated exoproducts, including proteases, exotoxin A, and pyoverdine itself.

There is a hierarchical organisation of iron regulation in *P. aeruginosa*, and different types of positive regulators were identified downstream of Pa-Fur, all responding to a signal originated by the siderophore itself: two component signal transduction systems, an AraC/XylS-like regulator, and a variety of FecIRA-like systems. Pa-Fur acts on top of the hierarchy, implying that all iron-repressible genes, including those encoding siderophore receptors, will be switched off in the presence of sufficient iron. Under iron-deficient conditions, Pa-Fur repression will be relieved and expression of siderophore receptors will occur, though at a ground level. Baseline expression of siderophore receptors is indeed essential for siderophore signalling (or for siderophore transfer to a secondary regulator) and, ultimately, for upregulation of transport genes upon siderophore binding to (or transport through) the receptor. Despite the different mechanisms at the basis of siderophore-dependent gene activation, a productive interaction between the siderophore and its receptor will always result in expression of siderophore uptake (and eventually biosynthesis) genes. In the special case of pyoverdine, such productive interaction will also trigger the PvdS-dependent production of three major pathogenicity factors that will finally cause tissue damage and facilitate iron supply during the infection either directly (pyoverdine) or indirectly (proteases and exotoxin A).

It must be acknowledged that a great deal of information on the molecular circuitry underlying the surface signalling process derives from the in-depth analysis of the Fec system of *E. coli* K-12. This system is still regarded as the archetypal surface signalling device, though relevant variations on the theme have recently been highlighted by the analysis of the FpvA/FpvR/FpvI-PvdS system of *P. aeruginosa*. Surface signalling in *P. aeruginosa* is characterised by remarkable peculiarities in terms of receptor activity, antisigma sensor specificity and energy coupling, and it will be mandatory to investigate in more detail the contribution of individual components to the divergent signal transduction pathway. In this context, the ability of FpvA to associate with both apo and ferric pyoverdine could be interpreted in the light of the dual specificity of FpvR. The existence of two distinct IS ECF sigma factors for either synthesis or transport of pyoverdine raises the possibility that these two processes undergo different signalling rules. It can be speculated that apo pyoverdine-loaded FpvA selectively activates PvdS and, consequently, synthesis of pyoverdine, proteases, and exotoxin A. The combination of these exoproducts would facilitate iron withholding and formation of ferric pyoverdine, which in turn could replace the FpvA-bound apo siderophore and cause induction of FpvI-dependent expression of FpvA and ferric pyoverdine uptake. According to this hypothesis, the iron-loaded status of pyoverdine would shift the recognition specificity of FpvR, resulting in differential activation of pyoverdine synthesis and uptake genes.

So far, surface signalling systems have been investigated in a limited number of bacterial species (reviewed in ref. [154]), though they were predicted to be widespread among prokaryotes. An extended search for putative IS ECF sigma factors and FecR-like sensor proteins in the NCBI microbial genome database indicated that FecIR homologues were present in at least 14 bacterial species belonging to 11 different genera, and up to 25 paralogues of such systems were detected in a single species[154]. Most of these systems were found in environmental, non-pathogenic genera like *Nitrosomonas*, *Pseudomonas*, and *Ralstonia*, consistent with the complexity of iron chelates present in the natural environments occupied by these bacteria. With the exception of *Bordetella perstussis* and *P. aeruginosa*, other pathogenic bacteria apparently had no such systems. Remarkably, the *fec* system is not present in the genome of enterohemorragic *E. coli* O157:H7 and in pathogenic enterobacteria[154]. *Pseudomonas* revealed a particularly wide repertoire of siderophore signalling devices, which reflects the life style of the species belonging to this genus. A genome-wide comparative analysis of FecR-like sensor and FecI-like ECF sigma factors in *P. putida* KT2440 and *P. aeruginosa* PAO1 evidenced a remarkable conservation of a certain subset of signalling systems, in particular those endowed with uptake specificities for ferric (di)citrate, ferrioxamine or still undefined siderophores[91]. This finding well

correlates with the overlapping ecological niche of *P. putida* and *P. aeruginosa* in natural environments. In contrast, the redundant, yet putative haem uptake systems (dependent on HasR, HxuC, OptI) and the *pigCDE* cluster had no equivalent in *P. putida* KT2440, suggesting that these system may be essential for *P. aeruginosa* parasitism and pathogenicity in the mammalian host[91].

At present, many questions concerned with the potential contribution of siderophore signalling to the virulence of *P. aeruginosa* remain unanswered. The evidence herein reported argues for a critical role of pyoverdine (and indirectly of signalling) in *P. aeruginosa* virulence (see Section 7). In the same vein as quorum sensing, pyoverdine signalling could delay production of virulence factors until the bacterial population has reached a sufficient density for successful spread in the host, thereby eluding recognition by the host's immune systems at an early and more vulnerable stage of infection. However, there is no evidence that pyoverdine signalling occurs in vivo, though indirect experimental data point to this direction. If this data will be confirmed in the future, then the pyoverdine signalling system could also be regarded as an ideal target for drugs capable of interfering with a critical step of *P. aeruginosa* pathogenesis.

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4

NEW INSIGHTS INTO THE BIOSYNTHESIS, MODE OF ACTION, AND REGULATION OF SYRINGOMYCIN, SYRINGOPEPTIN, AND CORONATINE

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1. INTRODUCTION

Pseudomonas syringae elicits a variety of symptoms on plants, including cankers, leaf spots, and galls, and is divided into pathogenic variants (pathovars; pv.) that vary in host range. The infection of host plants by *P. syringae* is a multi-step process involving epiphytic (surface) colonization, entry, establishment of infection sites in the apoplast (intercellular spaces), multiplication to high levels within host tissue, and production of disease symptoms[5]. The genetic basis of pathogenicity in *P. syringae* includes global regulatory loci[56, 108], the *hrp/hrc* cluster, phytotoxins, exopolysaccharides, and other virulence factors[5, 10, 19, 54]. The *hrp/hrc* genes encode a type III secretion system (TTSS), which is functionally similar to the TTSS identified in Gram-negative animal pathogens[38]. In *P. syringae*, the TTSS is required for growth

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in susceptible host plants and the activation of plant defense in nonhost plants; the latter is often associated with the hypersensitive response (HR)[123]. Although the TTSS is the best described virulence system for *P. syringae*, it is not the only component required for establishment of a successful infection[5, 54].

Many strains of *P. syringae* produce low-molecular weight, nonhost-specific phytotoxins that contribute to pathogenesis and induce chlorosis or necrosis[10]. Phytotoxins may be host-specific and exhibit the same specificity as the producing pathogen or nonhost-specific with a wider host range of activity than the producing pathogen. In general, the phytotoxins produced by *P. syringae* are nonhost-specific and cause symptoms on many plants which cannot be infected by the toxin-producing pathogen. This review will summarize our current understanding of the mechanism of action, biosynthesis, and regulation of the lipodepsipeptide (syringomycins, syringopeptins) and coronatine phytotoxins.

2. CORONATINE

The structure of coronatine (COR) is unusual and consists of two distinct components: (a) the polyketide coronafacic acid (CFA) and (b) coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine (Figure 1A)[61]. The structure and absolute stereochemistry of CFA were elucidated by X-ray crystallography, and the absolute stereochemistry of CMA was established by X-ray analysis of its N-acetyl derivative[60]. COR is generally the predominant coronafacoyl compound synthesized by COR producers and also the most toxic; however, other coronafacoyl compounds may be synthesized which contain various amino acids conjugated to CFA via an amide linkage[79].

2.1. Biosynthetic Route to Coronatine

Precursor feeding studies with ^{13}C-labeled substrates demonstrated that CFA is a novel polyketide synthesized from one unit of pyruvate, one unit of butyrate, and three acetate residues[96]. Little information is available regarding potential intermediates in the biosynthetic route to CFA, probably because such intermediates remain enzyme-bound. However, Mitchell *et al.*[81] identified a cyclopentenone compound, 2-[1-oxo-2-cyclopenten-2-ylmethyl]-butanoic acid (CPE), which may function as an intermediate or shunt product of the CFA biosynthetic pathway (Figure 2) . Results obtained from precursor incorporation experiments with (3,4-$^{13}C_2$)-glutamate, the isolation of CPE from the fermentation broth, and the organization of the CFA PKS allowed the creation of a hypothetical biosynthetic pathway for CFA (Figure 2).

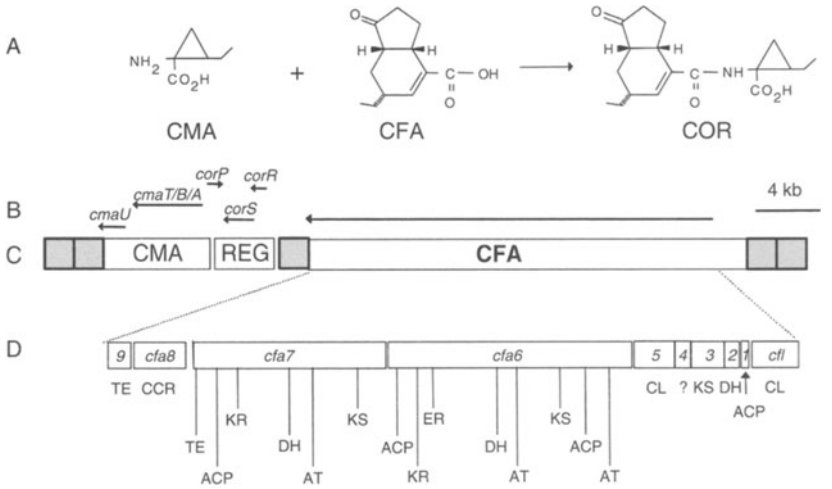

Figure 1. A. Structure of CMA, CFA, and COR. B. Transcriptional organization of the COR gene cluster in *P. syringae* pv. glycinea PG4180. C. Functional regions of the COR gene cluster in PG4180 indicating the location of the CMA structural gene cluster, a pathway-specific regulatory region (REG); and the CFA gene cluster. Shaded rectangles indicate the location of insertion sequences within and flanking the CFA and CMA/REG regions. D. Expanded view of the CFA biosynthetic gene cluster. Vertical lines show the catalytic domains in Cfa6 and Cfa7 (see Section 2.3.2).

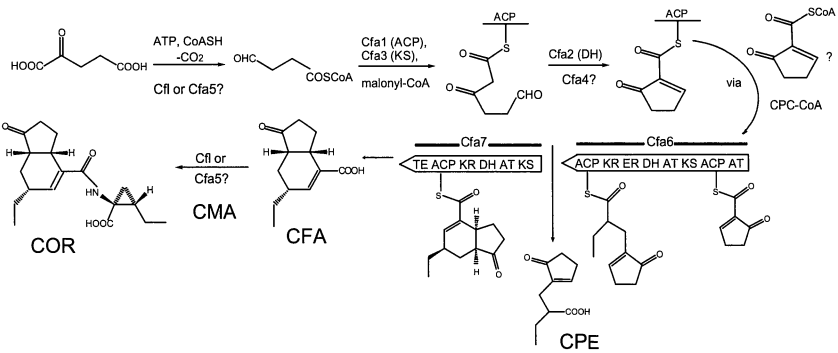

Figure 2. Hypothetical scheme for CFA biosynthesis showing potential functions of Cfl and Cfa1–Cfa7. Abbreviations: CFA, coronafacic acid, CMA, coronamic acid; CPE, 2-[1-oxo-2-cyclopenten-2-ylmethyl]-butanoic acid, and COR, coronatine.

The hypothesis postulates the oxidative deamination of glutamate to α-keto-glutarate followed by the loss of C-1 of α-ketoglutarate, which probably occurs via decarboxylation[63, 94]. The decarboxylation of α-ketoglutarate produces succinic semialdehyde (SSA) that would presumably be converted into its CoA ester before serving as a starter unit for polyketide assembly.

The isolation of CPE from the *P. syringae* fermentation suggests that a cyclopentenone ring is formed early in biosynthesis.

The biosynthetic route to CMA is also very speculative. Parry and coworkers[95] provided an important clue regarding the biosynthesis of CMA by demonstrating that L-alloisoleucine was a more immediate precursor to CMA than isoleucine. The current pathway to CMA acid involves the isomerization of isoleucine to form alloisoleucine and oxidative cyclization of alloisoleucine to form CMA. Further support for this biosynthetic route emerged from the evidence for CMA as a defined intermediate in the COR pathway[80].

The final step in the pathway to COR is presumed to be the ligation of CFA and CMA by an amide linkage. The enzyme(s) catalyzing this reaction is thought to lack rigid specificity for the amino acid substrate since a variety of coronafacoyl-amino acid conjugates have been isolated, including coronafacoylisoleucine, coronafacoylalloisoleucine, and coronafacoylvaline[79]. These amide conjugates of CFA are also phytotoxic, although at reduced levels when compared to COR.

2.2. Genetic Studies in Coronatine-Producing Bacteria

Production of the phytotoxin COR has been demonstrated in multiple pathovars of *P. syringae* including pvs. alisalensis, atropurpurea, glycinea, maculicola, morsprunorum, and tomato which infect broccoli, ryegrass, soybean, crucifers, *Prunus* spp., and tomato, respectively[10, 22]. In general, the COR gene cluster is plasmid-borne in pvs. atropurpurea, glycinea, morsprunorum, and tomato and chromosomally encoded in strains of pv. maculicola[13, 10, 111, 141, 151]; however, exceptions to these generalizations exist[87, 157]. Conjugal transfer of the plasmid-encoded COR gene cluster has been established for the COR plasmids in pvs. atropurpurea, glycinea, and morsprunorum, and tomato, respectively[4, 12, 111, 141]. The COR plasmids in these pathovars contain the origin of replication (*oriV*) and stability (*par*) loci previously identified in pOSU900, a cryptic plasmid originally described in *P. syringae* pv. syringae J900[89]. The *oriV* and *par* loci of pOSU900 are widely distributed in *P. syringae*[39, 118, 127].

2.3. Coronatine Biosynthesis in *P. syringae* pv. Glycinea PG4180

COR biosynthesis has been thoroughly investigated in *P. syringae* pv. glycinea PG4180, where the 32.8-kb COR gene cluster is borne on a 90-kb plasmid designated p4180A[11]. The involvement of p4180A in COR production was demonstrated by transforming this plasmid into *P. syringae* strains that do not produce COR. After acquiring the plasmid, *P. syringae* transformants

containing p4180A produced both CFA and COR, indicating that p4180A encodes all genes necessary for the biosynthesis of coronafacoyl compounds in *P. syringae*[11].

2.3.1. The CMA Gene Cluster in P. syringae PG4180

The nucleotide sequence of the 6.9-kb region containing the CMA biosynthetic gene cluster revealed the presence of four genes designated *cmaA*, *cmaB*, *cmaT*, and *cmaU*[18, 97, 133, 134] (Figure 1B). The CMA biosynthetic gene cluster was shown to encode two transcripts: one transcript was monocistronic and contained *cmaU*, and a second transcript was polycistronic and contained three co-transcribed genes designated *cmaA*, *cmaB*, and *cmaT* (Figure 1B). Start sites for both transcripts were determined by primer extension[133]. The deduced amino acid sequence of *cmaA* indicated that the enzyme contains an amino-acid-activating domain, whereas *cmaB* showed extensive homology with *syrB2*, a gene encoding an enzyme required for syringomycin synthesis[18, 52]. The deduced amino acid sequence of *cmaT* suggested that it functions as a thioesterase (TE), providing further support to the role of a thiotemplate mechanism for CMA biosynthesis[133]. CmaT was overproduced in *E. coli*, and assays using a variety of esters and thiolesters indicated that the overproduced protein was a functional esterase in vitro[97]. Recent analysis of the *cmaU* sequence has indicated relatedness to threonine efflux proteins, and it is tempting to speculate that this protein is either involved in the transport of CMA and or COR to the extracellular environment.

2.3.2. The Biosynthesis of CFA Involves Monofunctional and Multifunctional Polyketide Synthases

Polyketides exhibit an amazing degree of structural diversity and biological activity. Polyketide biosynthesis is similar to fatty acid biosynthesis, which begins with the condensation of acetyl-CoA (starter unit) and malonyl-CoA (chain extender unit), a reaction catalyzed by a ketosynthase (KS) to produce an enzyme-bound β-keto thiolester[55]. The keto group is then reduced to a methylene group in three steps: (a) reduction to a hydroxyl by a ketoreductase (KR); (b) dehydration to an unsaturated thiolester by a dehydratase (DH); and (c) a 1, 4 reduction to a saturated fatty acid by an enoyl reductase (ER). The intermediates in this process are bound to an acyl carrier protein (ACP) in the form of a thiolester. Unlike fatty acid synthases (FASs), polyketide synthases (PKSs) can make additional choices in the starter and extender groups, and the products vary in the extent of reduction. The PKSs of bacteria were originally classified into two types corresponding to the two common types of FASs. Type I PKSs consist of large, multifunctional proteins carrying domains for acyl transfer, an acyl carrier, condensation, reduction, dehydration, and enone reduction. Each domain is dedicated to a distinct step

in the biosynthetic reaction sequence and a collection of domains that catalyzes one round of condensation and processing has been termed a *module*. The best-characterized example of a Type I PKS is 6-deoxyerythronolide B synthase (DEBS), a PKS involved in the biosynthesis of erythromycin A[31].

The CFA transcript contains 10 discrete ORFs (Figure 1D). *cfa1*, *cfa2*, and *cfa3* are related to ACP, fatty acid DH, and β-ketoacyl synthase, respectively, whereas *cfl* and *cfa5* are similar to acyl-CoA ligases[73, 100]. The translational products of *cfa6* and *cfa7* show similarity to DEBS proteins, indicating that multifunctional PKSs also participate in CFA synthesis[106]. *cfa8* and *cfa9* (Figure 1D) show relatedness to crotonyl-CoA reductases and TEs, respectively. The nucleotide sequence of the CFA biosynthetic gene cluster resulted in a scheme incorporating the activities of both mono- and multifunctional PKS proteins (Figure 2).

A gene potentially involved in the ligation of CFA and CMA via amide bond formation was sequenced and designated *cfl* (*co*ronafacate *l*igase) (Figure 1D)[73]. Cfl is most closely related to enzymes that activate carboxylic acids by adenylation; consequently, this enzyme may catalyze the adenylation of CFA and the ligation of the CFA-adenylate to CMA. Coronafacate ligase has been overproduced in *E. coli* and *P. syringae* in soluble form[24, 73, 107]. However, efforts to demonstrate the ligation of CFA and CMA using purified Cfl in vitro have been unsuccessful; therefore, the assignment of a ligation function to this enzyme remains questionable.

2.3.3. Pathway-Specific Regulatory Genes for COR Production in PG4180

The structural genes for CFA and CMA biosynthesis are located on opposing ends of the COR gene cluster in PG4180 and are separated by a region containing the regulatory genes *corR, corS*, and *corP* (Figure 1B, C). The translational products of *corP* and *corR* show relatedness to response regulators in two-component regulatory systems, whereas *corS* shows similarity to histidine protein kinases (HPKs)[135]. The protein products of *corRPS* have been overproduced in *P. syringae* pv. glycinea PG4180, which made it possible to purify these proteins from their native host and examine their role in signal transduction. Transcriptional fusions, gel retardation assays, and DNaseI footprinting experiments demonstrated that CorR functions as a positive activator of COR gene expression and binds to the promoter regions of the *cfl*/*cfa* and *cmaABT* transcripts in PG4180[98, 140]. CorS was autophosphorylated by [γ-^{32}P]ATP (forming CorS~P) and has characteristics consistent with phosphorylation at a histidine residue[105]. Furthermore, the transphosphorylation of CorR by CorS~P was observed within 5 s, suggesting that CorR may be the cognate response regulator for CorS[105].

2.4. Global Regulators for Coronatine Biosynthesis and External Stimuli

2.4.1. σ^{54} is Required for Coronatine Gene Expression

σ^{54}, which is encoded by *rpoN*, is required for a variety of metabolic functions including the utilization of alternative carbon and nitrogen sources, nitrogen fixation, and the expression of virulence determinants[126]. *rpoN* mutants of *P. syringae* pv. *maculicola* ES4326 and *P. syringae* pv. glycinea PG4180 were nonpathogenic on *Arabidopsis* and soybean, respectively, and failed to initiate a HR in tobacco[3, 53]. Furthermore, *rpoN* was absolutely required for COR production and *cor* gene expression in *P. syringae* pv. maculicola ES4326 and pv. glycinea PG4180[3, 53]. Interestingly, the promoter regions of *cor* transcripts in PG4180 do not contain the −24(GG)/−12(GC) motif, which is the canonical sequence recognized by σ^{54}; this suggests that σ^{54} does not directly bind to these promoters. However, the *cfl*/*CFA* and *cmaABT* promoter regions in PG4180 contain motifs similar to the upstream activating sequences recognized by the σ^{54}-dependent activators, NifA and NtrC, respectively[73, 133]. Although the transcriptional start sites for the COR regulatory genes (*corR*, *corS*, and *corP*) have not been mapped, sequence analysis revealed several σ^{54} motifs ~500 bp upstream of the *corR* translational start[3]. Since CorR is a positive activator of *cfl*/CFA and *cmaABT* transcription in PG4180, σ^{54}-mediated expression of CorR provides a plausible explanation for *rpoN*-mediated control of *cfl*/CFA and *cmaABT* gene expression. Alternatively, σ^{54} may mediate *cor* gene expression indirectly through another regulatory gene, perhaps via a σ^{54}-dependent enhancer binding protein similar to NtrC or NifA.

2.4.2. Environmental Modulation of Coronatine Production

A variety of nutritional and environmental factors have been examined for their effect on COR production in *P. syringae* pv. glycinea PG4180[92]. Temperature had a highly significant effect on COR biosynthesis and *cor* gene expression in PG4180, and 18°C was an optimal temperature for both COR production and *cor* gene transcriptional activity[92, 135]. Rohde *et al.*[109] showed that COR production was thermoregulated in many strains of pvs. atropurpurea, maculicola, morsprunorum, and tomato, which may indicate that temperature is a common regulatory control for COR biosynthesis in other pathovars of *P. syringae*. However, it is interesting to note that strains of *P. syringae* pv. maculicola have been isolated that produce COR at relatively high temperatures (28–30°C)[143]. Smirnova *et al.*[119] have speculated that CorS might perceive temperature-mediated changes in the fatty acid composition of the inner membrane of the bacterial cell. This is an attractive hypothesis

because CorS contains six potential membrane-spanning helices[105], and one or more of these transmembrane regions may sense biochemical changes induced by temperature[119].

2.5. Coronatine Production by *P. syringae* pv. Tomato DC3000

Pseudomonas syringae pv. tomato DC3000, a pathogen of tomato, *Brassica* spp. (cabbage, cauliflower), and *Arabidopsis thaliana*[87, 139, 142], has become a model strain for investigating plant–microbe interactions, largely because of its genetic tractability, pathogenicity on *Arabidopsis*, and the availability of its genomic sequence (www.tigr.org). DC3000 and *P. syringae* pv. glycinea PG4180 differ in the amount of COR produced in vitro, the genomic location and organization of the COR genes, and the environmental cues for COR gene induction. Unlike many *P. syringae* pathovars where the COR genes are clustered and plasmid-encoded[10], the COR genes in DC3000 are chromosomally encoded, and the CFA and CMA structural genes are separated by a 26-kb region[19, 87]. Both clusters are bounded by IS-elements (transposases, integrases, and resolvases) presumably indicative of the mobile nature of this cluster between the chromosome and plasmid[19]. In DC3000, the COR structural and regulatory genes are highly homologous (90–98% nucleotide identity) to those previously described in *P. syringae* pv. glycinea PG4180. Interestingly, analysis of the CMA region of DC3000 revealed the possibility of four new genes with potential roles in COR synthesis[19].

Unlike *P. syringae* pv. glycinea PG4180, COR production by *P. syringae* pv. tomato DC3000 is induced in the presence of the host. Boch *et al.*[16] used in vivo expression technology (IVET) to identify genes that are specifically induced during the infection of *A. thaliana* by *P. syringae* pv. tomato DC3000. Approximately 15% of the genes induced during infection were localized to the CFA operon and included *cfl*, *cfa1*, *cfa6*, *cfa7*, *cfa8*, and *cfa9*[16]. These results are consistent with a previous study where a *cor*::*inaZ* transcriptional fusion was used to identify plant factors that stimulated *cor* gene expression in DC3000. Malic, citric, shikimic, and quinic acids were identified as compounds that stimulate COR production, and these compounds were present in leaf extracts and apoplastic fluids of tomato[72]. The importance of a "plant factor" in stimulating *cor* gene expression in DC3000 may help explain why DC3000 produces 25- to 40-fold less COR than PG4180 in vitro.

One of the most intriguing differences between DC3000 and PG4180 is that COR is required for the pathogenicity of DC3000 when the pathogen is either dip- or spray-inoculated to *A. thaliana* and tomato[82, 99]. However, when *A. thaliana* or tomato plants were infiltrated with DC3000 cells, COR was

not absolutely required for symptom development or the establishment of a successful infection[82]. Collectively, these results suggest that COR may have an integral role in facilitating the establishment of DC3000 in the apoplast of susceptible hosts. A recent study has provided evidence for differential regulation of COR biosynthesis in PG4180 and DC3000. In order to investigate this hypothesis, the *cfl* promoter regions from PG4180 and DC3000 were fused with the *uidA* gene, which encodes glucuronidase (GUS), and the strains were incubated at 21°C in COR-inducing media[139]. The kinetics of gene expression was clearly different in the two strains. In DC3000, the *cfl* promoter was activated within 6 hr after inoculation and maximal expression occurred prior to visible symptoms on both tomato and collard. However, in soybeans inoculated with PG4180, *cfl* promoter activity increased very slowly and was highest when visible chlorosis became apparent[139]. These results correlate well with a role for COR in the establishment of a successful infection for DC3000, whereas COR has a less prominent role in both symptom development and infection of soybean by PG4180.

2.5.1. The Type III Secretion System and COR Production in DC3000

The coordinated regulation of the TTSS and COR production has been addressed by analyzing COR production in defined *hrp*/*hrc* mutants of *P. syringae* pv. tomato DC3000. Despite the absence of a functional TTSS, COR was produced in vitro by mutants of DC3000 defective in *hrcC*, which encodes an outer-membrane protein required for type III-mediated secretion[21]. When inoculated to tomato plants, *hrcC* mutants produced chlorotic regions indicative of COR production, but lacked the necrotic lesions produced by the wild-type DC3000[99]. Furthermore, a DC3000 mutant containing a polar mutation in *hrcC*, which inactivates *hrcC*, *hrpT*, and *hrpV*, produced significantly higher amounts of COR than the wild-type strain in vitro. This mutant was able to produce COR earlier and at lower cell densities than the wild type. The results indicate that the *hrp*/*hrc* secretion system is not required for COR production, but mutations in this system may have regulatory effects on the production of virulence factors such as COR[99].

Further evidence for a regulatory connection between the TTSS and COR production in DC3000 was reported by Fouts *et al.*[36], who used a promoter-trapping screen to identify genes that are controlled by *hrpL*, which belongs to the family of extracytoplasmic sigma factors[146]. HrpL (σ^L) controls the activation of several transcripts in the *hrp/hrc* regulon, as well as *avr* genes, which modulate host range and fitness[57]. In the screen conducted by Fouts *et al.*[36], *hrpL*-regulated insertions were identified in two COR biosynthesis genes, *cfa1* and *cfa6*. This was an intriguing result, since neither *cor* gene contains a recognizable "*hrp*" box, a conserved motif in the promoter region of *hrpL*-dependent genes[147]. A Hidden Markov Model was utilized to look

for variations in functional Hrp boxes, with the goal of finding additional *hrpL*-modulated promoters in the DC3000 genome. This analysis indicated the potential existence of a "*hrp*" box upstream of *corR*, the response regulator known to control expression of both the CFA and CMA operons in PG4180[36]. Recent analysis of *corR* and *hrpL* mutants of DC3000 have suggested a role for both genes in the regulation of COR production in DC3000 (Raghavan and Bender, unpublished data).

2.6. Biological Effects of Coronatine

The most prominent symptom observed in leaf tissue treated with COR or infected with COR-producing strains of *P. syringae* is an intense spreading chlorosis[40]; this has been attributed to a loss of chlorophylls a and b in tomato and tobacco[66, 93]. Although chloroplasts in COR-treated tissue were significantly smaller, often had reduced starch deposits, and stained more intensely than chloroplasts in healthy tissue, the membranes were intact[93]. Immunolocalization experiments with COR-specific antiserum suggest that COR associates with chloroplasts in tomato leaves[152]. Recently, COR was shown to induce the expression of chlorophyllase, the first enzyme in the chlorophyll degradation pathway[14, 132]. Although the physiological significance of rapid chlorophyll breakdown is still unknown, it may be correlated with the rapid senescence of leaves exposed to COR[132].

In addition to chlorosis, COR induces an amazing array of effects in plants including anthocyanin production, alkaloid accumulation, ethylene emission, tendril coiling, the inhibition of root elongation, and hypertrophy[10, 34, 70, 144]. COR also induces volatile production and the accumulation of proteinase inhibitors, which are stress responses commonly associated with herbivory[34, 70, 92]. Other responses associated with COR include apoptotic cell death and the accumulation of flavonoid phytoalexins, responses which may be unique to grasses and other monocots[130, 150].

2.7. The Mode of Action for COR: Clues from Phytohormones

It has also been noted that COR acts as a structural and functional analogue of jasmonic acid (JA) and related signaling compounds such as methyl-JA (MeJA) and 12-oxo-phytodienoic acid (12-OPDA), the C_{18} precursor of JA/MeJA[34, 144]. JA and MeJA are plant growth regulators that are induced in response to biological stress; they also function as defensive molecules in response to herbivory and certain fungal plant pathogens[138]. When plants sustain damage at the plasma membrane, linolenic acid is converted to OPDA by

the sequential action of lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). OPDA is then further modified by a reductase and three β-oxidation steps, leading to JA. OPDA, JA, and other octadecanoid molecules can act upon various molecular targets to trigger gene expression and defense responses[70].

The identification of the *A. thaliana coi1* mutant that is insensitive to both jasmonates and COR supports the hypothesis that COR is a functional analogue of JA[34]. The wild-type *COI1* allele was localized in *A. thaliana* by mapped-based cloning, sequenced, and shown to encode a 66-kDa protein containing an N-terminal F-box motif and a leucine-rich repeat domain[148]. F-box proteins occur in eukaryotes and function as receptors that recruit regulatory proteins as substrates for ubiquitin-mediated degradation. COI1 participates in protein degradation via the E3 ubiquitin ligase complex, and this function could ultimately modulate the abundance of proteins that control the expression of JA/COR-responsive genes[29, 149].

2.7.1. Coronatine and Defense Signaling Pathways

Further clues regarding the mechanism of action for COR can be extrapolated from the interaction of JA with other hormone signaling pathways. In addition to the JA pathway, plant defense in response to microbial attack is regulated through a network that involves at least two other signaling molecules, salicylic acid (SA) and ethylene. SA plays a central role in plant defense, and the exogenous application of SA results in enhanced resistance to a broad range of pathogens[110]. Genetic studies have shown that SA is required for the rapid activation of defense responses that contain the growth of virulent pathogens and for the establishment of systemic acquired resistance (SAR), a heightened state of "plant immunity"[110]. The importance of SA in plant defense is further substantiated by the isolation of plant mutants that are impaired in SA production and transgenic plant lines that cannot accumulate SA because they express the SA-degrading enzyme, salicylate hydroxylase (NahG). Both SA-defective mutants and *nahG* transgenic plants exhibit enhanced disease susceptibility to a variety of pathogens, including *P. syringae*[90, 145].

The interactions between SA and JA signaling are complex; however, the primary mode of interaction between these pathways appears to be mutual antagonism[69]. The inhibitory effect of SA on the JA signaling pathway has been documented for both tomato and *A. thaliana*[30, 121]. However, there is evidence that a reciprocal negative interaction occurs via the JA-mediated antagonism of SA signaling[69]. For example, *coi1* (JA-insensitive) lines of *A. thaliana* show elevated resistance to *P. syringae* infection, and both symptom development and multiplication of bacterial populations are severely compromised[34, 68]. Furthermore, increased levels of SA and hyperexpression of the SA-dependent pathogenesis-related gene *PR-1* were observed in *coi1* plants

inoculated with *P. syringae* pv. tomato DC3000[68]. Given the importance of SA in mediating plant defense, these results imply a connection between COR and modulation of the SA pathway. The ability to inhibit or delay activation of SA-dependent host defense responses is likely to be an important trait of pathogenic bacteria, as this may provide a window of opportunity during which the pathogen can colonize the host tissue. Although the molecular mechanisms through which COR achieves this are not understood, additional clues have been provided by analysis of a JA-insensitive, COR-insensitive mutant of tomato (*jai1*). Like *coi1*, the *jai1* mutant also exhibits enhanced resistance to *P. syringae*, and resistance was correlated with a high level of *PR* gene expression and reduced expression of JA- and wound-response genes. These results suggest that COR may function to promote virulence by targeting the JA signaling pathway in tomato, with resulting antagonism of the SA pathway[153].

3. SYRINGOMYCINS AND SYRINGOPEPTINS

Pseudomonas syringae pv. syringae is a bacterial plant pathogen capable of causing disease on a large number of plant hosts throughout the temperate regions of the world[49]. Characteristically, *P. syringae* pv. syringae produces two classes of necrosis-inducing lipopeptide phytotoxins, the syringomycins and syringopeptins[10, 49, 59]. In addition, production of syringomycins and syringopeptins was recently reported in strains of *P. syringae* pvs. atrofaciens and lachrymans, indicating that production of these compounds is not limited to *P. syringae* pv. syringae[35, 136]. The syringomycins and syringopeptins exhibit significant structural and functional similarities, and both compounds contribute to virulence in bacterial–plant interactions[113]. The syringomycin and syringopeptin gene clusters are physically distinct, but are located adjacent to each other on the chromosome[113, 115]. They are both synthesized by a nonribosomal thiotemplate mechanism of biosynthesis and share a common secretory mechanism, and some aspects of regulation are also conserved[52, 74, 103, 113–115, 154, 155].

3.1. Structure of the Syringomycins and Syringopeptins

3.1.1. Structure of the Syringomycins

The lipodepsinonapeptide phytotoxins, which are generally referred to as the syringomycins, consist of a hydrophobic 3-hydroxy carboxylic acid tail and a charged cyclic peptide head containing nine amino acids[37, 117] (Figure 3). Three forms of syringomycin, which are designated SRA_1, SRE, and SRG, vary in the length of their fatty acid tail, possessing decanoic, dodecanoic, or tetradecanoic acid, respectively. The 3-hydroxy carboxylic acid tail is attached to an N-terminal serine via an amide bond. The ester linkage between the

N-terminal serine and the C-terminal residue, 4-chlorothreonine, forms a macrocyclic lactone ring. Interestingly, contained within this cyclic peptide are several rare amino acids, including 2,3-dehydroaminobutyric acid, 3-hydroxyaspartic acid, 4-chlorothreonine, and the D-isomers of serine and 2,4-diaminobutryic acid[112]. The length of the fatty acid tail does not significantly impact biological activity, but chlorination of L-threonine is essential[41]. There are several closely related lipodepsinonapeptides produced by different strains of *P. syringae* pv. syringae including syringotoxin, syringostatin, and pseudomycin (Figure 3). These lipodepsinonapeptides are

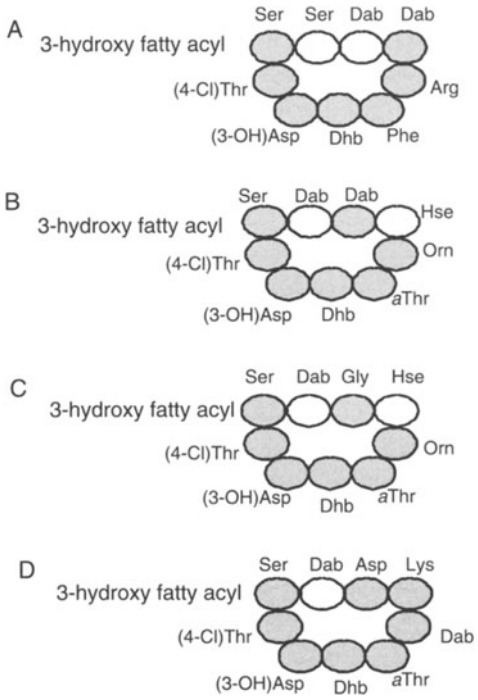

Figure 3. Structure of A. Syringomycin, B. Syringostatin, C. Syringotoxin, and D. Pseudomycin. The amino acid sequence of the lipodepsinonapeptides varies between positions 2 and 6. The 3-hydroxy fatty acyl group is a derivative of either decanoic acid (syringomycin), dodecanoic acid (syringomycin and syringostatin), tetradecanoic acid (all four lipononapeptides), or hexadecanoic acid (pseudomycin). L-isomers of the respective amino acids are denoted as shaded ovals, and D-isomers are denoted as white ovals. Abbreviations are Asp(3-OH), 3-hydroxyaspartic acid; Dab, 2,4-diaminobutryic acid; Dhb, 2,3-dehydroaminobutyric acid; *a*Thr, *allo*threonine; Hse, homoserine; Orn, ornithine; (4-Cl)Thr, 4-chlorothreonine; all other amino acids are identified by standard three-letter biochemical notation.

structurally similar, but contain different amino acid residues between positions two and six[10, 49].

3.1.2. Structure of the Syringopeptins

The syringopeptins constitute the second class of lipodepsipeptide phytotoxins produced by *P. syringae* pv. syringae. Depending on the strain of the bacterium, the syringopeptin produced will contain a peptide moiety of 22 or 25 amino acids. The peptide will be attached to a 3-hydroxydecanoic or 3-hydroxydodecanoic acid, resulting in forms A and B, respectively[7] (Figure 4). The syringopeptins contain a large number of hydrophobic amino acids, many of which are present in the D-isomeric form[8]. Three isoforms of syringopeptin 22 (SP_{22}) were identified and characterized, and these vary in amino acid composition[7, 46, 62] (Figure 4). Two isoforms of syringopeptin 25 (SP_{25}) are produced by strains of *P. syringae* pv. syringae, and these vary in their C-terminal amino acid[7, 112]. Interestingly, there is structural conservation among the various SP_{22} and SP_{25} toxins. In all isomers, the amino acid chain is cyclized to form a lactone ring via an ester bond between the C-terminal residue and the conserved *allo*threonine (*a*Thr)[7, 46, 62, 112].

Figure 4. Structures of the three isoforms of syringopeptin 22. A. SP_{22}; B. SP[SC]; and C. SP_{22} Phv. L-isomers of the respective amino acids are denoted as shaded ovals, and D-isomers are denoted as white ovals. For all three isoforms, the 3-hydroxylated fatty acid chain is 3-hydroxydecanoic or 3-hydroxydodecanoic acid for the A and B forms, respectively. Abbreviations are Asp(3-OH), 3-hydroxyaspartic acid; Dab, 2,4-diaminobutyric acid; Dhb, 2,3-dehydroaminobutyric acid; *a*Thr, *allo*threonine; Hse, homoserine; Orn, ornithine; (4-Cl)Thr, 4-chlorothreonine; all other amino acids are identified by standard three-letter biochemical notation.

3.2. Cytotoxicity of Syringomycin and Syringopeptin

Syringomycin and syringopeptin are nonspecific, necrosis-inducing phytotoxins. The amphipathic nature of the syringomycin and syringopeptin structures enables their insertion into the plant plasma membrane. This results in a transmembrane flux of K^+, H^+, and Ca^{2+}, disrupting the electrical potential across the plant cell membrane, and resulting in plant cell death[15, 88, 129]. As little as 50 ng/ml of either toxin can disrupt the membrane of tobacco protoplasts, inducing an influx of $^{45}Ca^{2+}$ (see ref. [58]). This suggests that these toxins are tremendously efficient; however, considering the relative size of the two toxins, it is evident that syringopeptin exhibits a greater toxic activity. This correlates with previous reports, indicating that syringopeptin is more effective than syringomycin in causing electrolyte leakage and plant cell necrosis[59, 71].

3.2.1. Pore Formation

Biophysical analysis of channel formation in planer lipids by syringomycin and syringopeptin enabled the characterization of the mechanism of pore formation[26, 58, 65]. The monomers insert into the membrane and quickly aggregate to form a pore, and the voltage-dependent behavior of ion channels indicate that at least 6 ($\pm$1) monomers of syringomycin and 3–5 ($\pm$1) monomers of syringopeptin were required to form pores[25, 26, 32, 65]. The pores formed by syringomycin contain the hydrophilic portion of the phytotoxin at the surface of the membrane and the hydrophobic portion in the bilayer[32]. Originally, syringomycin pores were described as having a pore radius between 0.6 and 1.0 nm[58]. Further investigation determined that syringomycin pores are not a fixed structure, and they can aggregate into clusters that have synchronous opening and closing[65]. Dalla Serra and colleagues[26] suggested that aggregations of syringomycin-induced pores could actually coalesce to form larger channels. Subsequently, it was determined that syringomycin pores possess a minimum radius of 0.6 nm and can increase to 1.7 nm as more monomers are added[26].

Early studies indicated that syringopeptin-induced pores are independent of concentration; suggesting that they do not aggregate as the concentration increases[25]. They are characterized as having a water-filled pore with the hydrophobic residues of the phytotoxin lining the pore[25]. Several researchers conducted studies to characterize the size and formation of syringopeptin-induced pores[2, 25, 26, 58]. However, the exact mechanism of syringopeptin-induced pore formation remains to be elucidated. It is clear that syringopeptin is able to form pores in pure black-lipid membranes and a similar mechanism was observed when plant membranes were used for channel formation assays[20]. Interestingly, while the two toxins may act together in the plant–pathogen interaction, there is no evidence that pores composed of both syringomycin and

syringopeptin monomers are formed[58]. Several models showing how these compounds facilitate pore formation have been published[57, 75].

3.2.2. Influence of Sterols on Phytotoxin Activity

Sterols influence the channel formation activity of both syringomycin and syringopeptin[26], but are not part of the channel structure[33]. Furthermore, different sterols exhibit distinct effects on the pore formation activity of the two phytotoxins[26]. Syringomycin and syringopeptin can form pores in artificial membranes that lack sterols[32, 58], but the addition of cholesterol (component of animal membranes) or ergosterol (component of fungal membranes) to lipid bilayers increased the pore-forming activity of syringomycin[26]. In addition, the presence of ergosterol promoted the binding of syringomycin to cells[64, 128]. Interestingly, syringopeptin does not require sterols but exhibited an increased pore forming activity when stigmasterol (a component of plant membranes) was added to the lipid bilayer[26]. Syringopeptin-induced pore forming activity was decreased when ergosterol was added and inactive when cholesterol was added to the lipid bilayers[26].

3.2.3. Influence of Sphingolipids on Phytotoxin Activity

Sphingolipids are a major lipid component of eukaryotic plasma membranes and are associated with many cellular processes, including functioning as mediators of stress responses and apoptosis[91]. Sphingolipids were shown to be associated with the sensitivity of the yeast, *Saccharomyces cerevisiae*, to syringomycin[23, 47]. It was observed that the resistance of a *S. cerevisiae* SYR2 mutant was due to the failure to produce 4-hydroxylated sphingolipids such as phytoceramide[47]. While the total sphingolipid content of the mutant was unchanged compared to the wild-type strain, the proportion of ceramide moieties was increased in the SYR2 mutant with dihydrosphingosine present, but not phytosphingosine. Further analysis identified that mutants defective in the elongation of N-acylated long chain fatty acids, attachment of the terminal phosphotidyl inositol group, or mannosylation, resulted in *S. cerevisiae* strains that were sensitive to syringomycin[125]. It was proposed that these modifications were important for efficient binding of syringomycin to the plasma membrane prior to channel formation[125]. It was also suggested that these enzymes encode modifications that may regulate the relative amounts of different sphingolipids in the membrane, either to facilitate or disrupt syringomycin pore formation[125]. Currently, the contribution of sphingolipids to syringopeptin pore formation is unknown.

3.3. Biosurfactant Activity

Syringomycin and syringopeptin both exhibit potent biosurfactant activities and can reduce the interfacial tension of water[26, 58]. Pure syringomycin

and syringopeptin preparations have a critical micelle concentration of approximately 1.2 mg/ml and 0.8 mg/ml, respectively[26, 58], suggesting that their surfactant activities are apparent at much lower concentrations. The biosurfactant activity resulting from these two phytotoxins may reduce the surface tension of water, thus enabling the bacterium to spread across plant surfaces[10].

3.4. Antimicrobial Activity

Syringomycin is not only phytotoxic, but also exhibits antimicrobial activity against a number of fungi, including *Geotrichum candidum* and *Rhodotorula pilimanae*[71, 120]. Many of these organisms are extremely sensitive to syringomycin, and concentrations as low as 0.8 μg/ml exhibit an inhibitory effect[120]. This sensitivity to syringomycin was conveniently exploited in the development of bioassays for syringomycin activity[50]. Other lipodepsinonapeptides such as syringostatin and syringotoxin exhibit comparable antifungal activity. The lipodepsipeptide syringopeptin exhibits antimicrobial activity against a number of bacteria and fungi but its spectrum of activity is distinct from that of syringomycin[71]. Syringopeptin is inhibitory to the Gram-positive bacterium, *Bacillus megaterium* and to some strains of the fungus *Botrytis cinerea*. Some of these microorganisms are quite sensitive and *B. megaterium* is often used as an indicator organism in syringopeptin bioassays[74, 113, 115]. It is important to note that the bioassays developed for the detection of syringomycin or syringopeptin take advantage of their differential spectrums of antimicrobial activity. *G. candidum* is resistant to syringopeptin and extremely sensitive to syringomycin; therefore, it is routinely used in syringomycin bioassays. In contrast, *B. megaterium* is more resistant to syringomycin and sensitive to syringopeptin, allowing for its use in syringopeptin bioassays[50, 74, 113, 115].

3.5. Contribution of Syringomycin and Syringopeptin to Virulence

The production of both syringomycins and syringopeptins is generally conserved in all strains of *P. syringae* pv. syringae, suggesting they are important in the plant–pathogen interaction. Both toxins induce necrosis and possess similar structures and functions; however, they differ in their actual contributions to the virulence of *P. syringae* pv. syringae[7, 59, 113]. Virulence assays using immature cherry fruits showed that *P. syringae* pv. syringae strains carrying mutations in the syringomycin (*syrB1*) or syringopeptin synthetase (*sypA*) genes were reduced in virulence 26% and 59%, respectively[113], and a *sypA-syrB1* double mutant was reduced 76% in virulence relative to the wild-type *P. syringae* pv. syringae B301D in immature cherry fruit assays[113]. The reduction in virulence of a *sypA-syrB1* double mutant was comparable to

that of the *syrD* mutant BR105[103], which is deficient in the secretion of both syringomycin and syringopeptin[44]. A *syrB1* mutant of *P. syringae* pv. syringae strain B728a did not exhibit a reduced virulence phenotype in pathogenicity tests using bean[67], suggesting that syringopeptin may exhibit a differential effect in the bean system. This indicates that the contribution of phytotoxins to virulence may vary with the plant–pathogen interaction.

3.6. Organization of the *syr-syp* Genomic Island

The syringomycin (*syr*) and syringopeptin (*syp*) gene clusters are distinct from each other and map adjacent to each other on the chromosome (Figure 5). This region encompasses greater than 2% of the genome and thus constitutes a genomic island in *P. syringae* pv. syringae strain B301D[115]. One of the most prominent features of the *syr-syp* cluster is the presence of extremely large genes (*syrE*, *sypA*, *sypB*, *sypC*) that encode peptide synthetases, as well as other genes (*syrB1*, *syrB2*, *syrC*), which are responsible for syringomycin and syringopeptin biosynthesis[52, 114, 154]. Nearly 70% of the *syr-syp* gene cluster encodes peptide synthetases. Furthermore, *sypC* spans 40.6 kb and is currently the largest prokaryotic ORF found in the GenBank/EMBL database[114]. Genes encoding functions for the regulation (*salA*, *syrF*, *syrG*, *syrP*) and secretion

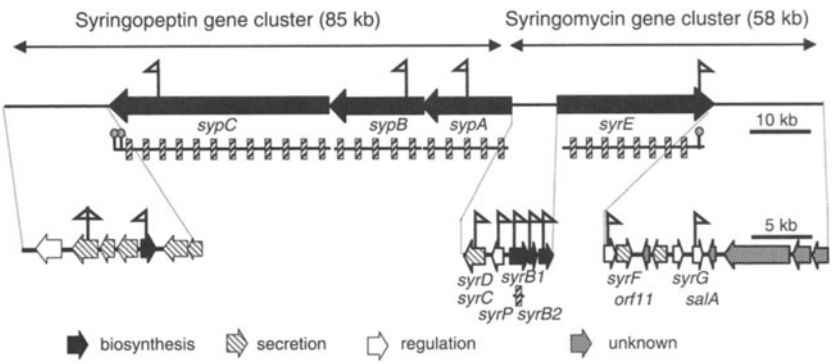

Figure 5. A physical map of the syringomycin and syringopeptin genomic island of *P. syringae* pv. syringae strain B301D. The black solid, diagonally lined, and open (not filled) arrows represent genes with predicted functions in the biosynthesis, secretion, and regulation, respectively, of syringomycin and syringopeptin. The arrows filled with diagonally hatched lines represent genes with unknown functions. The location of the amino acid activation modules for SypA, SypB, SypC, SyrE, and SyrB1 are located below their respective genes and are denoted as blocks with hatched lines. Vertical lines topped with a grey circle indicate TE domains. The disruption of genes designated with a flag pointed rightward indicate a loss of syringomycin production; a flag pointed leftward indicate mutants defective in syringopeptin production; a double-sided flag indicate mutants that are defective in both syringomycin and syringopeptin production.

(*syrD, sypD, orf11*) of syringomycin and syringopeptin have also been identified[74, 104, 155], and further contribute to our understanding of the organization and functional biology of the *syr-syp* genomic island.

3.7. Biosynthesis of Syringomycin and Syringopeptin Occurs via a Nonribosomal Mechanism of Peptide Synthesis

Biosynthesis of syringomycin and syringopeptin occurs via a nonribosomal, thiotemplate mechanism catalyzed by large multifunctional enzymes called peptides synthetases[42, 52, 113–115, 154]. A growing number of prokaryotic and eukaryotic organisms are found to synthesize peptides by nonribosomal peptide synthetases (NRPS). NRPS systems have been the focus of intense exploration for many years, and there are several excellent reviews covering this area of investigation[76, 86, 137]. Structurally, peptide synthetases are organized as repeated amino acid activation modules, encompassing conserved core motifs for condensation, aminoacyl adenylation, and thiolation (also known as peptidyl carrier) (Figure 5). One module is required for activation and incorporation of each amino acid contained within the peptide, and there is a colinear organization of the amino acid activating modules for the linear (type A) NRPS system, which is also reflected in the sequence of the amino acids within the peptide product[86].

3.7.1. Syringomycin Synthesis

The *syr* gene cluster is encoded by a ~58 kb region on the chromosome of *P. syringae* pv. syringae B301D (Figure 5). Two genes (*syrE* and *syrB1*) comprise 32.2 kb of this cluster and encode peptide synthetases. Mootz and colleagues[86] classified syringomycin biosynthesis as a nonlinear (type C) NRPS system, indicating that syringomycin biosynthesis does not follow the linear (type A) model directly, as the *syrB1* gene is located upstream of *syrE*[52]. Sequence analysis of the *syrE* gene (28.4 kb) revealed that it encodes eight amino acid activation modules, which contain domains for condensation, aminoacyl adenylation, and thiolation[52]. The aminoacyl adenylation domain, which controls substrate specificity region for the first amino acid activation module (SyrE-M1), was cloned and overexpressed to identify the amino acid recognized by this module[52]. In ATP-pyrophosphate (PP_i) exchange reactions, L-serine was recognized as a substrate, indicating that the first amino acid activated by SyrE is the N-terminal serine residue. A condensation domain precedes the aminoacyl adenylation domain, and is presumably responsible for the acyl transfer of the 3-hydroxy fatty acid from SyrC to the L-serine attached to SyrE-M1[27, 43, 124]. The activation and attachment of the next seven amino acids presumably occurs on SyrE-M2 to SyrE-M8 following the attachment of the fatty acid tail[52].

A TE domain is located on the C-terminal end of SyrE (Figure 5) and contains a GxSxG motif and the conserved aspartate and histidine residues that comprise the nucleophile elbow of TEs (Lu and Gross, personal communication). Presumably, the syringomycin peptide is transferred from the thiolation domain of SyrE-M8 by the 4′-phosphopantetheine cofactor to the TE domain, where the peptide forms a covalent bond with the conserved serine residue in the GxSxG motif[76, 86]. The syringomycin peptide could then be released by hydrolytic cleavage and subsequently cyclized. Unlike other peptide synthetases, the order of domains at the C-terminus of SyrE is unique. The TE domain is positioned between the ninth condensation and thiolation domains, but a corresponding aminoacyl adenylation domain is absent[52, 76].

The activation and incorporation of the ninth and final amino acid into the syringomycin peptide is likely catalyzed by SyrB1[154]. SyrB1 is encoded by the *syrB* operon, which contains SyrB1 (68 kDa) and SyrB2 (35 kDa). SyrB1 contains aminoacyl adenylation and thiolation domains, while SyrB2 is related to CmaB in the CMA structural gene cluster (Figure 2A)[18]. The region of SyrB1 that presumably controls substrate specificity for aminoacyl adenylation was compared with similar regions in other aminoacyl adenylation modules[113, 122, 154]. This analysis revealed that SyrB1 is likely to activate and incorporate L-Thr or Dhb, a dehydration product of L-[14C]-Thr[45]. PP_i exchange reactions using SyrB1 further demonstrated that SyrB1 activates L-Thr, but not other amino acids found within syringomycin such as 4-chlorothreonine or serine[52]. These results suggest that adenylated L-Thr is modified to 4-chlorothreonine, which results in the incorporation of 4-chlorothreonine into the syringomycin peptide[10, 52, 86]. Furthermore, PP_i exchange reactions using PvdD, a peptide synthetase involved in pyoverdin biosynthesis, revealed that PvdD specifically activated and incorporated L-Thr[1]. Three-dimensional structural modeling of the substrate-binding region of PvdD identified eight amino acid residues important in substrate specificity, and most of residues are present in SyrB1[1].

3.7.2. Syringopeptin Synthesis

The *syp* gene cluster spans ~85 kb and contains *sypA* (16.1 kb), *sypB* (16.3 kb), and *sypC* (40.6 kb), which each encode a peptide synthetase[113–115]. SypA, SypB, and SypC contain 5, 5, and 12 amino acid activation modules, respectively (Figure 5), which collectively account for the 22 amino acid residues that comprise syringopeptin SP_{22}[114]. Currently, there is no biochemical evidence to indicate the substrate specificity of the amino acid activation modules of the *syp* synthetases. Using the colinear model of NRPS, predictions were made regarding the specificity of the amino acid activation modules encoded by the *syp* genes[114]. This was followed by phylogenetic analysis of the substrate-binding pockets for the 22 *syp* and 9 *syr* amino acid

activation modules. This analysis revealed that the modules that incorporate identical or similar amino acids clustered together, indicating that syringopeptin is synthesized by a linear NRPS system[114].

The mechanism of syringopeptin synthesis can be postulated from a linear NRPS system, and SypA contains the first five amino acid activation modules. A condensation domain precedes the aminoacyl adenylation domain in SypA, suggesting that the former domain is involved in the acyl transfer of the 3-hydroxy fatty acid to the activated Dhb attached to SypA-M1[27, 124]. The activation and incorporation of the next four amino acids into syringopeptin should then follow, with translocation of the growing peptide from one module to the next. Presumably the N-terminal condensation domain of SypB-M6 interacts with SypA-M5 to transfer the growing peptide to SypB, where the next five amino acids are incorporated. The N-terminal condensation domain of SypC, SypC-M11, should then interact with SypB-M10 to transfer the growing peptide to the SypC. The growing peptide is then translocated from SypC-M11 to SypC-M22, and the last 12 amino acids are activated and incorporated into syringopeptin.

A unique structural feature of the syringopeptin synthetase is the presence of two potential TE domains at the C-terminal end of SypC. The first TE domain (260 amino acid residues) contains the complete "nucleophile elbow" with the GxSxG motif and conserved aspartate and histidine residues. The second TE domain (250 residues) is separated from the first domain by 11 amino acids and contains the conserved GxSxG and aspartate residues, but lacks the conserved histidine. Interestingly, TE domains are involved in the hydrolytic cleavage of surfactin and tyrocidine from their respective synthetases, a reaction that is followed by cyclization to form lactone rings[17, 28, 116, 131]. However, the significance of two TE domains in SypC and how these TEs might function to catalyze hydrolytic cleavage and cyclization of the syringopeptin remains unclear[114].

3.8. Regulation of Syringomycin and Syringopeptin Production

The regulation of syringomycin and syringopeptin is complex, and both plant signal molecules and nutritional factors impact production. Currently, the regulatory elements that control syringomycin are better characterized than those controlling syringopeptin, although studies suggest that both toxins share a similar regulatory cascade[41].

Syringomycin production is stimulated by plant signal molecules[84], including phenolic glycosides (e.g., arbutin), the flavonal glycosides (quercetin 3-runtinosyl-4′-glucoside and kaempferol 3-runtinosyl-4′-glucoside), and

dihydrowogonin 7-glucoside, a flavonone glucoside[83, 85]. When these signal molecules are present in low concentrations, the addition of D-fructose or sucrose can induce a 10-fold increase in transcriptional activity of a *syrB-lacZ* fusion[85]. Furthermore, strains of *P. syringae* pv. syringae exist that require the addition of arbutin and D-fructose to a defined media in order to produce syringomycin in vitro[104]. It is important to note that the signal molecules that stimulate syringomycin production are distinct from those that stimulate the *vir* gene operon in *Agrobacterium tumefaciens* and the *nod* genes in rhizobia[78, 102]. While plant signal molecules and sugars clearly induce syringomycin production, their effect on syringopeptin is unknown.

Two nutritional factors, iron and inorganic phosphate, impact syringomycin production[48]. Iron concentrations of 2 μM or higher are required for *syrB1-lacZ* transcriptional activity[83] and for maximal toxin production by strain B301D in vitro[48]. Interestingly, inorganic phosphate concentrations of 1 mM or higher inhibit syringomycin production[48], and a phosphate-associated regulatory element (*syrP*) has been identified[155].

3.8.1. SyrP Regulates Syringomycin Production

The mutation of *syrP* in *P. syringae* pv. syringae strain B301D results in a pleiotrophic response. The *syrP* mutant is insensitive to high inorganic phosphate concentrations, produces reduced amounts of syringomycin, and is reduced in virulence relative to the wild-type strain in immature sweet cherry fruits[155]. The *syrP* locus is physically located between the *syrD* and *syrB* operons (Figure 5) and encodes a protein with relatedness to CheA[156] and KinA[101], which are HPKs[6]. It has been suggested that *syrP* functions as a modulator in a phosphate-mediated phosphorelay system[155], which remains speculative as no other members of such a network are currently identified or characterized.

3.8.2. Regulatory Genes on the Right Border of the Syringomycin Gene Cluster

Three regulatory genes, *salA*, *syrF*, and *syrG*, are situated in the right border of the syringomycin gene cluster of *P. syringae* pv. syringae B301D[74] (Figure 5). The predicted protein products of these genes contain conserved C-terminal helix-turn-helix DNA-binding motifs and are related to the LuxR family of regulatory proteins. Although SalA, SyrF, and SyrG contain the conserved fingerprint of LuxR regulatory proteins, several conserved residues are absent, suggesting they may represent a new subgroup of the LuxR regulatory family[74]. A mutation in *salA* of *P. syringae* pv. syringae strain B301D completely eliminates syringomycin production, a finding that was also observed for a *salA* mutant of *P. syringae* pv. syringae B728a[67, 74]. A *salA* mutant of B301D was reduced 80% in virulence relative to the wild-type parent when inoculated to immature sweet cherry fruits, and *salA* was shown to positively

regulate the expression of a *syrB1::uidA* fusion. Although a *salA* mutant is reduced in syringopeptin production, the effect of a functional *salA* on the syringopeptin biosynthesis genes (*sypA*, *sypB*, *sypC*) is currently unknown[74].

A functional copy of *syrF* is critical for syringomycin production. A *syrF* mutant was 88% less inhibitory to *G. candidum* than the wild-type strain, and this was correlated with reduced expression of a *syrB1::uidA* fusion. Despite a strong effect on syringomycin production, a *syrF* mutant only exhibits a minor effect on syringopeptin production. A *syrG* mutant was reduced 40% in virulence in immature cherry fruit assays compared to a 26–30% reduction for a syringomycin biosynthetic mutant, suggesting that *syrG* may exert regulatory control over other unknown genes which may be involved in virulence[74].

The identification and characterization of *salA*, *syrF*, *syrG*, and the finding that *salA* is controlled by the *gacS*/*gacA* global regulators, have suggested the existence of a regulatory cascade that controls syringomycin production by *P. syringae* pv. syringae B301D[67, 74]. Although *gacS*/*gacA* are not physically linked to the syringomycin gene cluster, mutations in these genes exhibit profound effects on syringomycin production[56, 108]. Most of the intermediates in the regulatory cascade between *gacS*/*gacA* and syringomycin gene expression are unknown; however, disruption of *salA* in *P. syringae* pv. syringae B728a disrupts syringomycin production. Furthermore, overexpression of *salA* in a *gacS* mutant of B728a restores syringomycin, but not protease production[67], indicating that *salA* is downstream of the *gacS*/*gacA* regulon. Interestingly, *syrF* and *syrG* mutants have no observable effect on the expression of *salA*, indicating that *salA* functions upstream of *syrF*[74]. While *syrG* is associated with syringomycin production, the virulence data suggests that it may control other genes involved in the virulence of *P. syringae* pv. syringae strain B301D. The *salA* and *syrF* regulatory genes exhibit a much greater effect on syringomycin production than on syringopeptin production, suggesting that these two gene clusters share some common regulatory pathways.

3.9. Secretion of Syringomycin and Syringopeptin

It is apparent that *syrD* has a role in the secretion of both syringomycin and syringopeptin. The *syrD* gene is physically located between *syrP* and *sypA* in *P. syringae* pv. syringae strain B301D[113] (Figure 5). The predicted protein product of *syrD* is related to members of the ABC transporter family such as PvdE, which is responsible for pyoverdin secretion in *P. aeruginosa*[77, 103]. SyrD contains an ATP-binding pocket in its C-terminus, which is predicted to reside in the cytoplasm. The hydrophobic N-terminal portion of SyrD is inserted into the inner membrane[103]. A *syrD* mutant was significantly reduced

in virulence compared to the wild-type strain[103], and HPLC analysis determined that it was deficient in the secretion of both syringomycin and syringopeptin, suggesting a role for SyrD in the secretion of both phytotoxins[44]. Interestingly, a second putative ABC transporter, *sypD*, was identified in the left border of the syringopeptin gene cluster, but its role in phytotoxin secretion remains to be determined (Figure 5)[115]. Bioassay of *P. syringae* pv. syringae strain B301D.6, which carries a mini-Tn*5* transposon in this putative transporter, indicated that neither syringomycin nor syringopeptin was produced[115]. Further clues regarding secretion were provided by the identification of *orf11* in the right border of the syringomycin gene cluster[74] (Figure 5). The predicted protein of *orf1* gene is related to OprM, which comprises part of a prokaryotic type I secretion system in *P. aeruginosa*[51]. Disruption of *orf11* resulted in a strain that produced reduced zones of inhibition against *G. candidum* relative to the wild-type strain, suggesting less syringomycin was produced[74]. The effect of the mutated OprM homolog on syringopeptin was not evaluated.

4. CONCLUSIONS AND PERSPECTIVES

Biochemical studies have been completed or are underway to examine proposed functions for the protein products in the COR, syringomycin, and syringopeptin biosynthetic gene clusters. The models for toxin assembly discussed in this chapter represent "works in progress" and will inevitably undergo multiple revisions. The polyketide and peptide synthetases involved in the synthesis of these compounds represent a resource of genetic material that could ultimately expand the boundaries of antimicrobial metabolite synthesis. Activation of toxin synthesis is controlled by diverse environmental factors including plant signal molecules and temperature. Furthermore, some of the "global" regulatory genes for coronatine, syringomycin, and syringopeptin have now been identified. However, the mechanisms utilized for integration of these regulatory cascades and their cognate signals remain obscure and warrant further study.

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5

CURRENT IDEAS ON THE GENETICS AND REGULATION OF THE SYNTHESIS OF PHASEOLOTOXIN IN *PSEUDOMONAS SYRINGAE* PV. PHASEOLICOLA

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1. INTRODUCTION

The bacterium *Pseudomonas syringae* pv. phaseolicola is the causal agent of the "halo blight" disease of beans (*Phaseolus vulgaris* L.). This bacterium shares wide evolutionary relatedness with *Pseudomonas savastonoi*, and it was proposed that its taxonomic status be changed to *P. savastanoi* pv. phaseolicola; however, this proposal has been rejected and the organism has been maintained within the *P. syringae* group[66]. The disease attacks both foliage and pods, and is a major problem in temperate areas of the world. Leaf symptoms appear several days after infection as small water-soaked spots on the lower surface. At 7–10 days after infection, the lesions appear as greasy water-soaked points of infections. Pods can also be infected and lesions appear as brown or red water-soaked spots. Seeds may also become infected and the disease may be transmitted through the infected seed. At temperatures between

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18°C and 23°C and high humidity, a zone of yellow-green tissue may develop around the initial site of infection after 3–8 days, giving rise to what is known as the chlorotic halo. In cases of severe infection, the chlorosis may become systemic. In fact, halo blight is considered to be a low-temperature disease and epidemic potential is greatest at temperatures ranging between 18°C and 22°C.

Infested plant residues are the major source of *P. syringae* pv. phaseolicola inoculum. Bacteria from these residues are transported to neighboring healthy plant tissues by splashing rain or blowing soil particles. The pathogen gains entrance to the plants through natural openings such as stomata and hydathodes, through wounds during periods of high humidity or when free moisture occurs on the plant surface.

Pseudomonas syringae pv. phaseolicola can also be found as an epiphyte on the leaves of nonhost or susceptible plants and not develop any obvious disease symptoms. During secondary infections, these epiphytes may be transferred to other plants by rain splash or by contact between adjacent wet leaves after rain or dew. Blossoms, pods and stems can also be colonized under favorable conditions.

At the physiological level, plant tissues infected with *P. syringae* pv. phaseolicola show an accumulation of ornithine due to the inhibition of the enzyme ornithincarbamoyltransferase (OCTase; EC 2.1.3.3)[55] which catalyzes the conversion of ornithine to citrulline, a reaction common to both arginine biosynthesis and the urea cycle[46]. The toxin is mobile within the plant, moving to the apex and newly expanded leaves causing severe retardation of growth in these tissues and disrupting apical dominance[46], younger leaves being apparently more susceptible to halo symptoms, as long as the temperature is appropriate for the full development of symptoms. For more detailed information on the effect of the toxin on the plant, the reader is referred to an excellent review by R. Mitchell[45].

Pseudomonas syringae pv. phaseolicola (Burkholder) is a Gram-negative rod-shaped bacterium. It is strictly aerobic, has a negative reaction for oxidase and arginine dihydrolase and produces a diffusible fluorescent pigment in iron-deficient media. Bacterial growth on standard media such as King's medium B (KB) produces white to cream-colored colonies. Its optimal temperature for growth is 28–30°C being unable to grow at temperatures above 30°C. Essentially all the *phaseolicola* pathovars produce a nonhost-specific toxin known as phaseolotoxin when grown at 16–20°C, although the degree of toxin production may vary within different isolates[33].

2. PHYTOTOXINS

Phytopathogenic bacteria within all genera have been reported to produce phytotoxins[15], but the mechanisms of action of these secondary

metabolites have only been elucidated for just a few of them such as tabtoxin, coronatin, syringomycin and related lipodepsinonapeptides and phaseolotoxin[6]. Phytotoxins can be defined as compounds synthesized by the pathogen during pathogenesis, that are deleterious to the host. In the vast majority of cases, bacterial phytotoxins increase disease severity, that is, they act as virulence factors—a quantitative trait. However, in very few instances, phytotoxins produced by bacteria have been shown to be required for disease production, that is, to act as pathogenicity factors—a qualitative trait having to do with the ability to cause disease[15, 68].

Bacteria usually produce the toxin and one or more analogues of it. However, the active component is non-variable, and the variable portion of the analogue does not interfere with the action of the toxin, or is cleaved off by hydrolytic enzymes before the toxin interacts with its target[42]. It is also possible that a single phytotoxin could have more than one molecular target since multiple targets with the same catalytic reaction center may interact with the toxin, or there may be different targets with catalytic mechanisms which share the same or similar binding sites for the toxin[15].

The toxins that are produced by plant pathogens can be divided into two broad classes: host-specific toxins which damage or destroy plant tissues susceptible to the toxin-producing organism, but have little or no effect on other plants; and nonspecific toxins which are toxic to both hosts and nonhosts of the pathogen. Most of the extracellular toxins produced by phytopathogenic bacteria have been found to be nonspecific.

3. PHASEOLOTOXIN

This chapter will focus primarily on the genetics and regulation of phaseolotoxin production, and it is not intended to be an exhaustive analysis on the chemical structure of phaseolotoxin, or the biochemical mechanisms involved in its synthesis. However, some of these aspects will be discussed in the context of their relevance to this chapter.

Pseudomonas syringae pv. phaseolicola and *P. syringae* pv. actinidiae, a pathogen of kiwi fruit, produce a nonhost-specific, chlorosis-inducing toxin known as phaseolotoxin[17, 44, 65, 73]. Application of crude, toxin-containing culture filtrates from this bacterium to leaves of beans and to leaves of a variety of other plants result in the formation of chlorotic haloes[17]. The production of this toxin is temperature dependent, being optimally produced at 18–20°C, while no detectable amounts of the toxin are present at 30°C[23, 43, 52]. As mentioned before, the toxin inhibits the enzyme OCTase which catalyzes the conversion of ornithine to citrulline[55] leading to a phenotypic requirement for arginine in the tissues affected. This toxin is active not only against plant OCTases but also against bacterial and mammalian enzymes[17, 47]. This has led

to the development of a rapid bioassay that measures growth inhibition of a bacterial culture exposed to this toxin[71].

Phaseolotoxin has been shown to be a competitive inhibitor of OCTase from *Escherichia coli* with a Ki of 0.2 μM with respect to carbamoylphosphate. The inhibition is noncompetitive with respect to ornithine with a Ki of 0.9 μM[45].

3.1. Chemical Structure

Phaseolotoxin is composed of two readily identifiable moieties: the inorganic moiety, N^{δ}-N'-sulfodiaminophosphynil and the L-ornithyl-alanyl-homoarginine tripeptide. A possible function for this tripeptide may be to facilitate export of the toxin, since uptake into *E. coli* and *Salmonella typhimurium* has been shown to be mediated by an oligopeptide permease[70]. However, the tripeptide does not seem to be necessary for translocation or uptake into plant cells[47].

In the plant phaseolotoxin, N^{δ}-(N'-sulfodiaminophosphynil)-L-ornithyl-alanyl-homoarginine, is hydrolyzed removing the alanyl-homoarginine residues to produce N^{δ}-(N'-sulfodiaminophosphynil)-L-ornithyl (PSorn), also known as octicidine, which is a more potent inhibitor of OCTase[37, 74]. The biosynthesis of citrulline proceeds through a transition state intermediary of the reaction between carbamoyl phosphate and ornithine. PSorn has high structural homology with the proposed transition state molecule and has been proposed to be a transition state analogue that binds tightly, but reversibly to OCTase[37] (Figure 1).

3.2. Immunity of *P. syringae* pv. phaseolicola to Phaseolotoxin: The *argK* Gene

Pseudomonas syringae pv. phaseolicola is insensitive to the effect of its own toxin. The reason for this immunity was attributed to the presence of a phaseolotoxin-resistant OCTase (ROCT) activity in strains of *P. syringae* pv. phaseolicola capable of producing the toxin[18, 72]. Under the non-permissive condition for the synthesis of phaseolotoxin, that is, growth at 28°C, extracts of *P. syringae* pv. phaseolicola were found to contain a phaseolotoxin-sensitive OCTase (SOCT). However, it was not clear whether the different OCTases were the product of different genes or a single enzyme with different susceptibility due to post-transcriptional modifications[72].

Pseudomonas syringae pv. phaseolicola was later shown to have two detectable OCTase activities, one resistant and one sensitive to the toxin, with the resistant activity being detectable at 18–20°C but not at 30°C[34, 35].

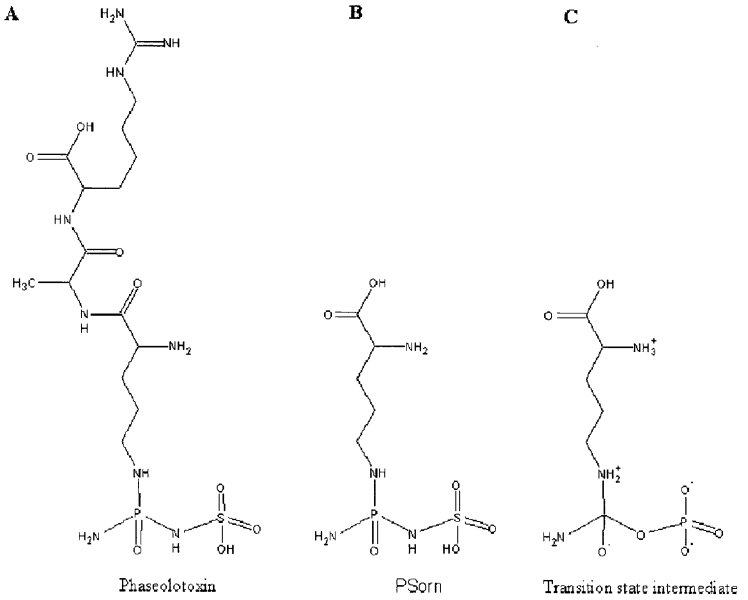

Figure 1. Chemical structure of: A. Phaseolotoxin N^{δ}-(*N'*-sulfodiaminophosphynil)-L-ornithyl-alanyl-homoarginine, B. PSorn N^{δ}-(*N'*-sulfodiaminophosphynil)-L-ornithine, and C. Proposed OCTase reaction mechanisms transition state.

The insensitivity of the ROCT was found to be due to a lower affinity for carbamoylphosphate and a slower binding of ornithine. The enzyme, however, will still efficiently catalyze the synthesis of citrulline given a sufficiently high concentration of ornithine[75].

Of the two possible explanations that could account for the two OCTase activities detected, the one postulating two different genes was shown to be correct when two different DNA fragments encoding OCTase were cloned from *P. syringae* pv. phaseolicola. These fragments did not cross-hybridize, could complement OCTase deficient strains of *E. coli* and *P. aeruginosa*, and encoded OCTases that differed in their sensitivity to phaseolotoxin[57].

The gene of *P. syringae* pv. phaseolicola coding for the ROCT was later isolated and characterized. The phaseolotoxin-resistant OCTase activity is the product of the *argK* gene. This enzyme is a polypeptide composed of 327 amino acid residues with a molecular mass of 36.52 kDa. Comparison of the amino acid sequence of the product of *argK* to the product of the *E. coli argF* gene, coding for a phaseolotoxin-sensitive OCTase, reveals two significant changes in two highly conserved regions implicated in substrate binding. One of these changes results in having Gly in the ROCT, the product of *argK*, instead of the Thr present in the SOCT, the product of *argF*, that has been implicated in carbamoyl phosphate binding and/or catalysis[48].

A second mechanism of self-protection has been proposed based on the observation that wild-type *P. syringae* pv. phaseolicola grown at 28°C, thus producing only the phaseolotoxin-sensitive OCTase, is resistant to exogenous application of the toxin[16].

3.3. Biosynthesis of Phaseolotoxin

Nothing is known at the moment regarding the synthesis of the inorganic moiety N^{δ}-(*N'*-sulfodiaminophosphynil) of phaseolotoxin and no precursors have yet been identified. However, the synthesis of the unusual amino acid homoarginine (HArg) present in the phaseolotoxin molecule was postulated to involve an enzyme with amidinotransferase (AT) activity, and biochemical activity corresponding to an AT was indeed detected in extracts of *P. syringae* pv. phaseolicola[40, 59]. The AT activity detected was shown to catalyze the transfer of an amidino group from arginine to lysine to produce one molecule of homoarginine and one molecule of ornithine (Figure 2), both being precursors in the biosynthesis of phaseolotoxin. This activity was shown to be closely related to phaseolotoxin biosynthesis because of the following observations: (a) The highest rates of enzyme activity corresponded with the highest rates of phaseolotoxin production during the growth cycle. (b) A mutant of *P. syringae* pv. phaseolicola producing low amounts of the toxin exhibited low levels of AT activity. (c) Only those pathovars of *P. syringae* known to produce phaseolotoxin had any detectable AT activity, and (d) the highest AT activity was observed when cultures were grown at 18°C and the lowest was observed at 30°C following the temperature-dependent production of phaseolotoxin. Further characterization of this AT indicated an apparent mol. wt. of 200 kDa and a K_M of 7.7 mM for arginine and 4.2 mM for lysine[40, 59].

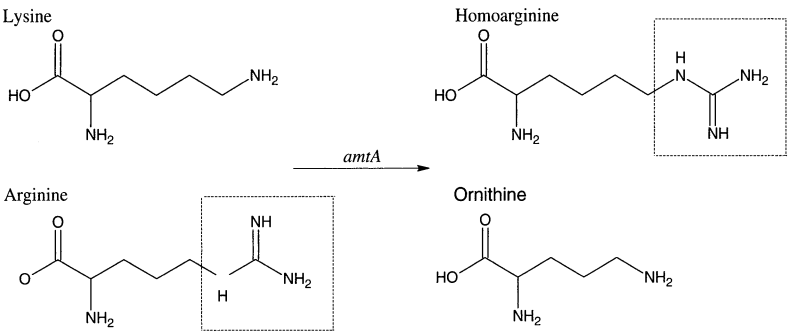

Figure 2. Biosynthesis of homoarginine mediated by the amidinotransferase enzyme encoded by *amtA* of *P. syringae* pv. phaseolicola.

Amidinotransferases catalyze the transfer of an amidino group from L-arginine to guanidines. L-arginine:glycine amidinotransferases have been shown to be involved in the biosynthesis of creatine in vertebrates (humans, rats and pigs). In plants of the Fabaceae family, such as *Galega officinalis* and *Lathyrus sativus*, L-arginine:glycine AT activity has been detected, and in *G. officinalis* this enzyme is involved in the synthesis of guanidino acetic acid, a precursor of galegine. In microorganisms, AT activity has been reported in strains of *Streptomyces* known to synthesize streptomycin or hydroxystreptomycin. This activity is involved in the biosynthesis of *N,N'*-diamidinostreptamine-P, one of the precursors of streptomycin[40, 77].

Amidinotransferases are well conserved showing over 80% sequence similarity among the StrB1 proteins from *Streptomyces griseus, S. galbus* and *S. glaucescens*. This overall similarity decreases when any of the StrB1 products is compared to StrB2, and it further decreases when compared to mammalian amidinotransferases. Nevertheless, in both mammalian and bacterial amidinotransferases, specific Cys, His and Asp residues have been implicated in substrate binding and are conserved among the different AT sequences available, with the only exception being StrB2 of *S. griseus*, which seems to lack enzymatic activity[20, 30, 31].

The high degree of conservation among amidinotransferases allowed the possibility of designing specific primers for PCR-amplification of sequences of *P. syringae* pv. phaseolicola corresponding to an AT. Using this strategy, it was possible to amplify a 718 bp specific fragment from total DNA from *P. syringae* pv. phaseolicola which was then used as a probe for the identification of clones carrying the chromosomal region containing the complete gene of this bacterium, which was shown to correspond to a sequence coding for a polypeptide of 362 residues (41.36 kDa), with around 40% sequence similarity to L-argine:inosamine-phosphate amidinotransferase from three species of *Streptomyces*, and 50.4% with an L-arginine:glycine amidinotransferase from human mitochondria. Furthermore, the Cys, His and Asp residues involved in substrate binding were all conserved. Expression of *amtA, argK* and phaseolotoxin production were also shown to occur at 18°C but not at 28°C. A role for *amtA* in the synthesis of phaseolotoxin was conclusively shown by interrupting this gene in vitro, exchanging this allele in the wild-type *P. syringae* pv. phaseolicola, and demonstrating that this mutant lacks the capacity to synthesize phaseolotoxin under permissive conditions[29]. Furthermore, this gene has only been detected in strains of *P. syringae* pv. phaseolicola, and has been shown to be present relatively close to the *argK* gene on the chromosome of this bacterium suggesting the presence of a phaseolotoxin-gene cluster[29].

3.3.1. Synthesis of the Ornithine-Alanine-Homoarginine Tripeptide

It has been suggested that a nonribosomal thiotemplate mechanism similar to that described for the synthesis of peptide antibiotics may be used

by *P. syringae* pv. phaseolicola to produce the ornithyl-alanyl-homoarginine tripeptide. In this case, the enzymes involved, generically called "peptide synthetases," can be produced by bacteria and fungi, are usually clustered in large operons and are composed of specific modules or domains related to activities such as thiolation and adenylation[14, 69] that are well conserved among different peptide synthases.

This conservation has allowed the use of degenerate primers to detect genes coding for this type of enzyme in different organisms[8]. Using such a general method based on the conservation of the peptide synthetases, a PCR amplified fragment from *P. syringae* pv. phaseolicola was obtained and assumed to be part of a putative phaseolotoxin synthetase[76]. However, unpublished data from our laboratory suggested that what had been amplified was a region of the *pvdD* gene involved in pioverdine biosynthesis, which also involves a peptide synthetase mechanism[41].

The fact that the approach used with *P. syringae* pv. phaseolicola allowed the identification of a peptide synthetase, but failed to amplify a specific fragment of the postulated phaseolotoxin synthetase, may suggest that the enzymes involved in the synthesis of the tripeptide may somehow differ from the model peptide synthetases that have been well characterized.

As mentioned before, *P. syringae* pv. phaseolicola has the capacity to produce phaseolotoxin analogues, two analogues isolated together with phaseolotoxin were found to lack the homoarginine residue normally found in the tripeptide and which may be replaced by a 2-serine analogue or by as yet unidentified residues. However, it was concluded that production of analogues of phaseolotoxin is uncommon and not likely to be strain specific but rather an abnormality within a strain[42].

4. GENETICS OF PHASEOLOTOXIN

A chromosomal gene cluster involved in phaseolotoxin production, secretion and/or immunity has been identified through transposon mutagenesis. In this report, 5,180 presumptive Tn*5* insertion mutants were screened using a microbiological assay and initially 6 putative Tox^- mutants were isolated from *P. syringae* pv. phaseolicola. Complementation analysis of these mutants with a cosmid library allowed the identification of a clone, pRCP17, carrying genes involved in phaseolotoxin synthesis[56]. Further studies with pRCP17 indicated that *argK* was also present in this cosmid, and that the genes present in this cosmid were all present in a 22 kbp *Kpn*I fragment on the chromosome of *P. syringae* pv. phaseolicola[57].

In an independent study, a second clone was isolated which contained a 25 kbp insert and complemented several Tox^- mutants. This clone, designated

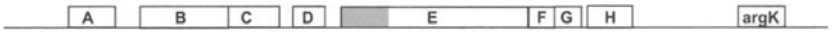

Figure 3. *pht* cluster of *P. syringae* pv. phaseolicola. The cluster includes regions contained in both pRCP17 and pKH120. A, B, C, D, E, F, G and H are complementation groups described by Zhang *et al.* The distance between *argK* and *phtH* is not known. The grey box indicates the location of the gene coding for the fatty acid desaturase.

pHK120, was shown to contain eight transcriptional units which were designated as *phtA* through *phtH*. Both clones pRCP17 and pHK120 were shown to partially overlap, however, pHK120 lacked *argK* but contained sequences missing in pRCP17[79] (Figure 3).

Sequence analysis of a 2.6 kbp *Eco*RI fragment localized within the *phtE* locus indicated the presence of a gene with homology to a fatty acid desaturase[26]. Further analysis of this same locus confirmed the presence of this gene and also indicated the presence of six ORFs within *phtE* that seemed to be part of a single transcriptional unit. Besides the homology with the desaturase gene present in ORF6, ORF3 was shown to have homology with acetylornithine aminotransferase, which is involved in ornithine synthesis, and ORF5 presented homology to a transcriptional factor of the AraC family[80].

The *amtA* gene corresponding to the amidinotransferase has been located to a 270 kbp *Pme*I fragment of the chromosome of *P. syringae* pv. phaseolicola which also harbors *argK*[12, 29], however, the physical distance between *amtA* and *argK*, as well as the relative position of these genes to the *pht* locus has yet to be resolved.

5. REGULATION OF THE SYNTHESIS OF PHASEOLOTOXIN

It had been observed that Tox^- mutants of *P. syringae* pv. phaseolicola were also impaired in the synthesis of the ROCT suggesting a coordinated response of genes coding for enzymes involved in the synthesis of phaseolotoxin, and the *argK* gene coding for the ROCT[15, 57]. Soon it was well established that the biosynthesis of phaseolotoxin, as well as expression from *argK* were temperature dependent occurring between 16°C and 20°C.

Regulation of toxin production mediated by temperature has also been reported in *P. syringae* pv. glycinea regarding the synthesis of coronatine, and in fact coronatine in this bacterium occurs in the same temperature range as phaseolotoxin in *P. syringae* pv. phaseolicola, that is, phytotoxin production is optimal at 18°C and negligible at 30°C (see the review by Bender and

Scholz-Schroeder and Bender *et al.*[6]). Coronatine is also produced by some strains of *P. syringae* pv. atropurpurea, maculicola, morsprunorum and tomato, and in every case toxin production is thermoregulated[60].

Several genetic elements have been identified and implicated in regulation of coronatine production, among them, the product of *rpoN*, σ^{54} (see refs [2], [27]), and a modified two-component regulatory system composed of *corR, corS* and *corP*, with the product of *corR* being a positive regulator of *cor* gene expression[58].

5.1. Regulation of *argK* Expression

In *P. syringae* pv. Phaseolicola, thermoregulation appears to be negatively regulated with phaseolotoxin production being the consequence of relief of repression rather than induction, and three lines of evidence support this idea: (a) minimal clones containing the *argK* gene expressed constitutively when transferred to *E. coli*[48], (b) the promoter sequence of *argK* presents a −35 and −10 region characteristic of the negatively controlled Pribnow-promoters which are recognized by the housekeeping σ^{70} (see ref. [25]), and (c) multiple copies of a DNA fragment containing a defined sequence, when introduced into *P. syringae* pv. phaseolicola bypassed thermoregulation suggesting titration of a repressor molecule by a defined sequence in the DNA. These specific sequences have also been identified in *argK*[61].

The putative sequence responsible for the titration effect, designated thermoregulatory region, (TRR) has a core motif G/C AAAG repeated once with a 5 bp spacer between repeats[61, 62]. Four similar sequences can be found in *argK* located between the transcription start site and the start codon, and through mobility shift assays these have been shown to interact with a protein present only at 28°C in extracts from *P. syringae* pv. phaseolicola. Nevertheless, multiple copies of this sequence have been shown to be unable to override thermoregulation presumably due to a weak interaction between the TRRs of *argK* and the TRR-binding protein[62].

In an attempt to search for the putative repressor molecule involved in repression of phaseolotoxin synthesis and *argK* expression in our laboratory, we sought to obtain an *argF*$^-$ mutant lacking the SOCT, which we thought would behave as an arginine auxotroph when cultured at 28°C, since under these conditions *argK* would be repressed. A second round of Tn-mediated mutation of this *argF*$^-$ mutant followed by selection on minimal media would in principle allow us to recover mutants where the putative repressor had been inactivated. Therefore, null mutants were constructed in *argF* by interruption with a Tc^R cassette. However, none of the mutants were arginine auxotrophs and Northern-blot data indicated that *argK* was being expressed and producing the ROCT and thus, supplying the arginine required for growth at 28°C[38].

Why was *argK* being derepressed at 28°C? We had previously postulated that a precursor of phaseolotoxin, or the toxin itself could act as an inducer that would bind to the postulated repressor molecule when cells were grown at 18°C relieving repression[48]. We also had observed that *argK* was being expressed in the *amtA* mutant[38], which indicated that homoarginine could be ruled out as the inducer since this molecule is not produced by this strain, and ornithine and alanine, the other two amino acids present in the organic moiety, were not likely candidates because of their ubiquity. A molecule that could fulfill this role could be the inorganic moiety of phaseolotoxin, the N^{δ}-(*N'*-sulfodiaminophosphinyl) group. The chemical structure of this molecule strongly resembles the structure of carbamoyl phosphate, and, as mentioned before, this molecule when bound to ornithine to form N^{δ}-(*N'*-sulfodiaminophosphinyl)-L-ornithine (PSOrn) results in a transition state analogue of an unstable intermediate in the reaction calalyzed by OCTase[37].

We assumed that in the *argF*$^{-}$ mutant grown at 28 °C, lacking SOCT, the reaction to synthesize citrulline from carbamoyl phosphate and ornithine would not proceed and these precursors would then accumulate. Could derepression of *argK* be induced by the accumulated carbamoylphosphate? To test this idea, wild-type *P. syringae* pv. phaseolicola was grown in minimal medium at 28°C. As expected, *argK* was not induced under these culture conditions; however, upon addition of carbamoyl phosphate to the medium, *argK* was derepressed even though the temperature was kept at 28°C. Under these conditions, phaseolotoxin could not be detected, indicating that carbamoylphosphate has an effect only on the *argK* gene but not on those genes involved in biosynthesis of the toxin.

This result strongly suggests the following: (a) *argK* is subject to induction with negative control, where the inducer molecule could be N^{δ}-(*N'*-sulfodiaminophosphinyl) and (b) *argK* is not directly regulated by temperature but coordination with phaseolotoxin synthesis is mediated through the synthesis of the inducer which occurs at the lower permissive temperatures.

These results indicate that *argK* is not directly thermoregulated whereas some other genes involved in the synthesis of phaseolotoxin seem to be more stringent in their dependence to temperature for expression. Therefore, it may be possible that more than one repressor protein is found in cells growing at 28°C, one species responding to temperature changes, acting on genes involved in phaseolotoxin synthesis and able to recognize the TRR motif as binding sites, and a second species, a repressor molecule acting on the *argK* gene with presumably an allosteric site for recognition of the inorganic moiety of phaseolotoxin, and only a limited affinity for sequences similar to the TRR motif.

In Gram-negative bacteria, regulation of the *argF* gene has been shown to be mediated by a repressor molecule involved also in the regulation of the

synthesis of carbamoyl phosphate. Could this repressor molecule play any role in the biosynthesis of phaseolotoxin in *P. syringae* pv. phaseolicola?

5.2. Role of the ArgR Regulatory Protein

In Gram-negative bacteria, the regulation of arginine biosynthesis is tightly regulated because this pathway is closely related to the pathways of biosynthesis of pyrimidines and polyamines. In bacteria such as *E. coli, argF*, the gene coding for the anabolic OCTase is being negatively regulated through the product of *argR*, a DNA-binding protein that specifically recognizes a specific sequence within *arg*-related operons called "arg-boxes."

The regulation of arginine biosynthesis is of considerable interest in Gram-negative bacteria because of the occurrence of a common precursor to the biosynthesis of arginine and the pyrimidines—carbamoyl phosphate. Another important metabolic interconnection is the role of arginine and ornithine as precursors in the biosynthesis of polyamines.

The biosynthesis of arginine in *E. coli* proceeds in eight consecutive steps starting from glutamate. The first step is the synthesis of N-acetyl-glutamate from glutamate and acetyl-CoA. The four subsequent steps deal with acetylated intermediates, and the biosynthetic strategies can vary within different genera of microorganisms. From ornithine onward, the intermediates of the pathway appear to be common to all organisms. In both *E. coli* and *S. typhimurium*, the synthesis of the eight enzymes is repressed by arginine to different extents, and a single regulatory gene, *argR*, accounts for this repression in both organisms.

Carbamoyl phosphate is a precursor common to the synthesis of arginine and the pyrimidines, and in *E. coli* and *S. typhimurium* it is produced by the enzyme carbamoyl phosphate synthase (CPS) which uses glutamine as the physiological amino group donor. In both organisms, CPS is cumulatively repressed by arginine and the pyrimidines, and again, the arginine repressor, ArgR, is involved. Polyamine biosynthesis has been extensively studied in *E. coli*. The diamine putrescine can be synthesized directly by decarboxylation of ornithine or indirectly by decarboxylation of arginine into agmatine followed by hydrolysis of agmatine into putrescine and urea by an agmatine ureohydrolase. Putrescine can then give rise to spermidine and cadaverine (spermidine is normally not found in *E. coli*)[11, 22].

In *Pseudomonas*, the "Arg box" has not been observed in the *arg* genes, although a repressor molecule, called ArgR, has been involved in the regulation of some of the genes required for arginine biosynthesis[53, 54]. In *P. aeruginosa*, feedback inhibition of the first two enzymes of the arginine biosynthetic pathway, as well as the critical role played by the intermediate metabolite N-acetylglutamate in this control, ensures a sensitive and immediate

control of arginine synthesis[22]. The sixth step involves the enzyme OCTase, encoded by *argF*, which is the only arginine biosynthetic enzyme whose synthesis is repressible by exogenous arginine together with the repressor protein, ArgR[54]. ArgR of *P. aeruginosa* is a member of the AraC/XylS family of regulatory proteins[9, 53], and bears no similarity with ArgR characteristic of enteric bacteria or *B. subtillis*, which agrees with the fact that ArgR from *E. coli* does not control the expression of *argF* from *P. aeruginosa*[10].

ArgR is a global regulator required for the expression of genes involved in arginine catabolism (*aru*), arginine uptake and transport (*aot-argR* operon) as well as *gdhB* and the two-component system CbrA-CbrB involved in induction of *argR*[39, 49, 50, 54]. In *Pseudomonas*, the *argR* product is a repressor of the *argF* and *carAB* operons involved in arginine and carbamoyl phosphate biosynthesis[53, 54].

In our laboratory, the argR gene from *P. syringae* pv. phaseolicola has been isolated and characterized[28]. It consists of 1,065 nucleotides, and the translation product corresponds to a polypeptide of 354 amino acids with an Mr of 39.4 kDa. This gene presents very high similarity to *argR* from *P. aeruginosa* (54.5% at the nucleotide level and 81.5% at the amino acid level), and to transcriptional regulatory proteins reported for several *Pseudomonas* species and other Gram-negative bacteria. This relationship was also supported by the finding of a double Helix-Turn-Helix (HTH) domain[9] characteristic of bacterial DNA binding regulatory proteins of the AraC/XylS family[21, 53].

Partial sequencing and analysis of the 5′ end of *argR* in *P. syringae* pv. phaseolicola indicated the presence of a truncated ORF similar to the *aotP* gene of *P. aeruginosa*. In *P. aeruginosa*, the *argR* gene is the last gene in a polycistronic operon comprising six open reading frames, out of which five correspond to proteins involved in arginine and ornithine transport (*aot*) and the last being *argR*. Thus, the operon structure *aotJQMOPargR* present in *P. aeruginosa* may be repeated in *P. syringae* pv. phaseolicola[28, 50].

In *P. aeruginosa*, ArgR negatively controls the expression of *argF* and also acts as an inducer of genes required for the catabolism of arginine[54], the same phenotype was observed for *P. syringae* pv. phaseolicola[28]. ArgR exerts its repressor activity by binding to the *argF* promoter, both in *P. aeruginosa* and *P. syringae* pv. phaseolicola. However, DNA retardation experiments clearly showed that while ArgR binds to the promoter region of *argF* in *P. syringae* pv. phaseolicola, it does not bind to *argK*[28, 54].

The lack of binding of ArgR to the *argK* promoter region correlated with the fact that phaseolotoxin production, *argK* expression and normal pathogenicity could occur in *P. syringae* pv. phaseolicola in the absence of ArgR.

It could be argued that the lack of an effect by the ArgR regulator on *argK* expression could have been predicted because the ArgR binding domain ($TGTCGCN_8AA$) present in the promoter region of *argF*[54] differs

substantially from the core motif (G/C AAAG) of the TRR binding domain identified in *argK*[62]. However, the *argK* region also contains a DNA sequence similar to the core of the binding site of ArgR (TGTCG) located 80 bp upstream of the ATG initiation codon. Furthermore, the TRR domains identified in the *argK* region may not be involved in its regulation since, as mentioned before, this gene is not directly regulated by temperature but most likely through induction mediated by a precursor of phaseolotoxin resembling carbamoyl phosphate[38].

6. FOREIGN ORIGIN OF THE PHASEOLOTOXIN GENE CLUSTER

In *P. syringae* pv. phaseolicola, the nucleotide sequence of *argK* has very low similarity with the sequence from *argF*, so low in fact that these genes do not cross-hybridize[48, 57]. The sequence of *argK* also presents a G+C content of 49.4%, below that of *argF* (57.3%) and the *hrp* genes (56–58%). These characteristics are indicative that in *P. syringae* pv. phaseolicola, *argK* did not evolve from the house keeping gene *argF*, but rather seems to have been acquired horizontally from another microorganism. The close linkage of *argK* to some of the other genes involved in the synthesis of phaseolotoxin, and its essential role in protection against this toxin also suggests that not only *argK*, but all the genes involved in phaseolotoxin biosynthesis and immunity may have been acquired together[25].

Pseudomonas syringae pv. actinidiae possesses an *argK* gene identical to that of *P. syringae* pv. phaseolicola; however, analysis of the sequence of the spacer regions between genes encoding 16S and 23S rRNAs indicate that these pathovars evolved divergently from each other during phylogenetic evolution. This in turn suggests that the *argK*-Tox cluster was transferred recently during evolution to the genomes of *P. syringae* pv. phaseolicola and pv. actinidiae[65].

Phylogenetic analysis of different pathovars of *P. syringae* suggests that *hrpL* and *hrpS* genes have always been stably maintained in the genome without exchange among the different pathovars of *P. syringae*. This analysis reveals that *P. syringae* pv. phaseolicola and pv. actinidiae are localized in different evolutionary groups which supports the idea that the *argK*-tox gene cluster was obtained horizontally after the pathovars had separated[64]. Furthermore, mapping the *argK*-tox gene cluster on the physical map of the genomes from *P. syringae* pv. phaseolicola and pv. actinidiae shows a different arrangement of these genes with respect to *argF* and the *hrp* genes.

A phylogenetic analysis of genes coding for OCTase in different organisms places the *argK* gene close to *argF* from *Lactobacillus plantarum*, a Gram-positive species with a G+C content similar to that of *P. syringae* pv. phaseolicola[63].

6.1. Pathogenicity Islands in *P. syringae* pv. Phaseolicola

Many pathogenicity factors (and antibiotic-resistance genes) that are plasmid encoded are often found adjacent to each other, and often flanked by repetitive sequences or transposable elements. Recent studies suggest that pathogenic bacteria evolved from related nonpathogenic organisms by acquiring relatively large blocks of genetic material encoding pathogenicity and virulence factors rather than by slow, adaptive evolution of preexisting genes. A very large number of essential virulence determinants, particularly toxins and adherence factors, are found on mobile genetic elements, which can be disseminated to other bacteria by bacterial conjugation, transformation and transduction[19].

In many cases, virulence genes are clustered in large contiguous blocks found as chromosomal inserts or pathogenicity islands (Pai). These islands are often flanked by sequences that suggest that the DNA segment was acquired through an event of illegitimate recombination that resembles transposition or phage insertion. In fact, these DNA clusters also are often inserted into hot spots in the chromosome, and the sequences within the Pai are not homologous to those found in a nonpathogenic strain, but the sequences immediately adjacent to the Pai are found in pathogens and nonpathogens[19].

Pai have been identified in many bacterial pathogens such as uropathogenic and enteropathogenic *Escherichia coli, Helicobacter pylori, Yersinia enterocolitica, Shigella flexneri* and *Salmonella* species. However, only one or two Pai have been identified so far in any one of these bacteria, except for Salmonella, for which up to four pathogenicity islands have been described[7, 19, 78]. It has also been noted that pathogenicity islands vary with respect to the overall G+C content of the chromosome of the bacteria harboring them, again suggesting that these DNA fragments correspond to relatively recent acquisition of genetic material through lateral transfer.

In the case of the plant pathogenic bacteria, Pais have been described in *P. syringae* pv. phaseolicola[32], and in pathovars syringae and tomato[3, 5], and in *Xanthomonas campestris* and *Xanthomonas axonopodis*[36, 51]. These Pai have been shown to carry *vir, avr* and *hrp* genes. So far there has been no report of Pai containing genes coding for the synthesis of phytotoxins. However, there is genetic evidence that in *P. syringae* pv. phaseolicola genes required for phaseolotoxin synthesis and immunity are clustered[56, 57, 79]. The construction of a physical map of the chromosome of *P. syringae* pv. phaseolicola[12], allowed the identification of a single *Pme*I fragment of 270 kbp carrying *argK* and *amtA* genes[29], which strongly suggests that the complete set of genes required for phaseolotoxin synthesis and immunity may be clustered in this region of the chromosome, and suggests also that this whole cluster is the region which may have been received through horizontal gene transfer during evolution. A similar case has been reported for *P. syringae* pv. syringae B301D, in which

the genes required for syringomycin and syringopeptin are clustered together in a defined region of the chromosome[67]. It has also been suggested that genes involved in phytotoxin synthesis in both pathovars may constitute a Pai[29, 67].

7. PERSPECTIVES

One question that remains unanswered concerns the events related to the detection and signal transduction implicated with the temperature response. It is interesting to note that a fatty acid desaturase has been reported as part of a gene cluster related to phaseolotoxin synthesis[26, 80] and a mutant in this desaturase was defective in phaseolotoxin synthesis[73]. Furthermore, fatty acid desaturases have also been implicated in response to cold shock in Gram-positive species[1, 24]. Whether a desaturase is implicated in thermosensing remains to be elucidated, as well as to whether this desaturase is controlled by a two-component system homologous to *desK-desR* of *Bacillus subtilis*[1] that in *P. syringae* pv. phaseolicola could be part of the gene cluster related to phaseolotoxin synthesis.

A working model for the induction of genes involved in phaseolotoxin synthesis in *P. syringae* pv. phaseolicola growing at 18°C has been proposed[38] that includes the following steps (Figure 4):

1. Temperature down-shifts may be sensed through the physical state of the membrane, implicating changes mediated by a desaturase, somehow the signal is processed and transduced to some effector molecule that will act upon the repressor of phaseolotoxin genes (R_1 in Figure 4).

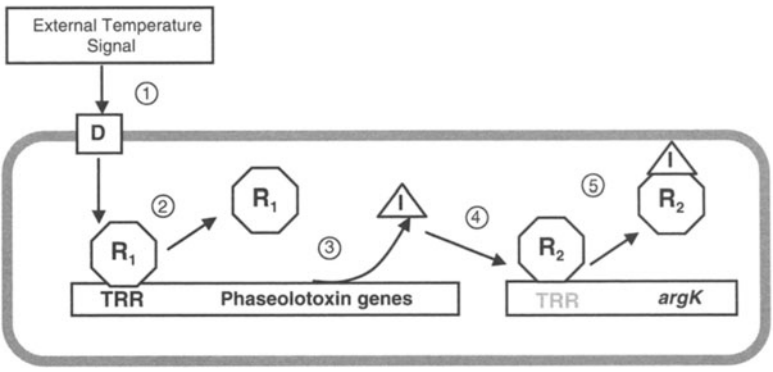

Figure 4. Proposed model of the regulation of genes involved in phaseolotoxin synthesis and immunity in *P. syringae* pv. phaseolicola.

2. At 28°C, genes involved in the synthesis of phaseolotoxin, such as *amtA*, are being negatively regulated by a repressor molecule (R_1) that may bind to TTR motifs, when down-shifted to 16–18°C the signal coming from the temperature sensing system relieves repression and these genes are actively expressed.
3. The gene products from the phaseolotoxin genes synthesize both the inorganic (I) and peptide moiety of phaseolotoxin.
4. The inorganic moiety resembling carbamoyl phosphate acts as an inducer of *argK* by binding to an *argK*-specific repressor molecule (R_2) and removing it from its DNA target sequence.
5. Expression of *argK* takes place and a ROCT is synthesized to provide arginine, homoarginine and ornithine via the amidinotransferase reaction, for cell growth and phaseolotoxin synthesis.

Is phaseolotoxin a virulence factor? Phaseolotoxin has been postulated to be a virulence factor, because it seems to be responsible for an increase in disease severity but not necessary for the onset or establishment of the disease. This has been suggested by the reports of analysis of naturally occurring or mutagen-induced phaseolotoxin-deficient (Tox^-) mutants[23]. However, in several cases it has been clear that mutants initially selected as Tox^- have turned out to still be capable of producing low amounts of the toxin[56]. However, under conditions that allow *P. syringae* pv. phaseolicola to infect and develop halo-blight-like symptoms in tobacco plants, a transgenic tobacco plant which has been transformed with the *argK* gene of *P. syringae* pv. phaseolicola, and efficiently expresses the phaseolotoxin-resistant OCTase, not only becomes immune to the toxin, but is also capable of developing a hypersensitive response to the otherwise compatible pathogen *P. syringae* pv. phaseolicola[13]. This and a similar observation in the tobacco-*P. syrinagae* pv. tabaci system[4] suggest that toxins such as phaseolotoxin and tabtoxin may actually be playing a role in the establishment of the disease and not only in the severity of the symptoms, therefore acting more like pathogenicity factors rather than virulence factors.

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6

THE TYPE III SECRETION SYSTEMS OF PLANT-ASSOCIATED PSEUDOMONADS: GENES AND PROTEINS ON THE MOVE

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1. INTRODUCTION

Pseudomonas syringae and *Pseudomonas fluorescens* are plant-associated bacteria that have very different relationships with plants. *P. syringae* is a foliar pathogen that affects a wide range of crop plants, whereas *P. fluorescens* is a commensal that colonizes the rhizosphere and can even benefit plants as a plant growth-promoting rhizobacterium (PGPR). The type III secretion system (TTSS), which is capable of injecting virulence effector proteins into plant cells, is central to the pathogenicity of *P. syringae* and is also present in some strains of *P. fluorescens*. The TTSS is thus a logical entry point for exploring the diversity and evolution of plant-associated pseudomonads and the molecular basis for their differing interactions with plants.

The genetics and activities of the TTSS in *P. syringae* and *P. fluorescens* are highly dynamic in that genes associated with the TTSS appear to have been horizontally acquired and are often in highly variable regions of the genome, and that the TTSS directs the coordinated movement of a variety of proteins

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across multiple bacterial and host barriers to specific targets in host cells. This chapter will address the evolutionary movement of the TTSS genes in *P. syringae* and *P. fluorescens* and the movement of effector proteins through the TTSS pathway. Other chapters will address the genomes of *P. syringae* and *P. fluorescens*, the lives of these bacteria in the phyllosphere and rhizosphere, respectively, and the phytotoxins and global regulators that are also important to the virulence of *P. syringae*.

1.1. The Importance of the TTSS in Bacterial Pathogenesis

The TTSS is key to the pathogenicity of many Gram-negative pathogens of plants and animals[36]. The importance of the TTSS to *P. syringae* is indicated by the prevalence of mutants that are defective in the pathway among random mutants isolated on the basis of diminished pathogenic interactions with test plants[94]. *P. syringae* TTSS genes were originally designated *hrp* because they are required for elicitation of the hypersensitive response (HR) on nonhost plants and pathogenicity (or parasitism) on host plants[93]. A subset of nine TTSS genes encoding conserved, core components of the machinery were later renamed as *hrc* (*hrp c*onserved) and given the last-letter designation of their *Yersinia ysc* homologs[18]. Most *P. fluorescens* strains are non-pathogenic, and *P. fluorescens* TTSS genes were initially identified in screens for *P. fluorescens* mutants that fail to elicit the HR, and in functional genomic screens for plant-induced genes (Preston *et al.*, 2001). Due to the latter phenotype, and because *P. fluorescens* TTSS genes have not been shown to have a role in pathogenesis, the TTSS genes of *P. fluorescens* SBW25 have been named *rsp* and *rsc*, for *r*hizosphere-induced *s*ecretion *p*athway.

As discussed below, the *P. syringae* Hrp TTSS injects parasite effector proteins into plant cells. The genes encoding these proteins are primarily designated *avr* (*avi*rulence) or *hop* (*H*rp *o*uter *p*rotein), based on the phenotype by which they were discovered[4]. The Avr phenotype is observed when an effector gene from one race or pathovar of *P. syringae* is heterologously expressed in another strain, resulting in elicitation of strong defenses associated with the HR and pathogen "avirulence" in an otherwise susceptible host plant[87, 140]. The HR is a programmed cell death that is triggered by detection of the effector (or its activity) by a cognate resistance (*R*) gene product within plant cells, and it is part of a gene-for-gene system that determines the interactions between pathogen races and host cultivars[85]. Although the TTSS is typically essential to the pathogenicity of *P. syringae*, mutations in individual effector genes frequently have little or no phenotype, apparently due to redundancy among the effectors. The identification of effector genes in *P. syringae* genome sequences and the activity of effectors in plant cells will be discussed further in another chapter. Here, we will focus on the TTSS pathway that carries these important parasite proteins to their host targets.

1.2. Biology of *P. syringae* and *P. fluorescens*

P. syringae strains are noted for their diverse and host-specific interactions with plants[71, 72]. A specific strain may be assigned to one of at least 50 pathovars based on its host range among different plant species and then further assigned to a race based on differential interactions with cultivars of the host[43]. Many *P. syringae* strains are effective epiphytes[72]. As a pathogen, *P. syringae* typically enters the leaves through stomates, multiplies for several days in leaf intercellular spaces, and then produces necrotic lesions that are commonly surrounded by a phytotoxin-induced chlorotic halo[72]. Interactions with a nonhost plant or a resistant cultivar of the host frequently elicit the HR.

The TTSS appears central to the pathogenic abilities of *P. syringae*, but three observations highlight the complex pathobiological context in which the TTSS operates. First, *P. syringae* pv. syringae B728a mutants deficient in GacA (a two-component regulator of the production of phytotoxins and other virulence factors) can grow as well as the wild type in host bean leaves but without producing the necrotic lesions characteristic of brown spot disease[74, 162]. Second, B728a TTSS mutants, although unable to grow in inoculated plants in the laboratory and strongly reduced in growth on plants in the field, occasionally achieve high populations on bean leaves in the field and cause typical lesions[70]. Third, mutation of a single effector gene, *hopPtoM*, in *P. syringae* pv. tomato DC3000 enables the bacterium to grow as well as the wild type in host tomato leaves but without causing the lesions normally associated with bacterial speck disease[8]. Evidently, the role of the TTSS in *P. syringae* interactions with plants is complex, and there may be alternative mechanisms that can contribute to growth and symptom production in different strains and situations. The recently completed genome of DC3000 contains 50 confirmed or putative TTSS effector genes, nearly 300 genes implicated in virulence, more than 800 unknown genes that are not found in *Pseudomonas aeruginosa* or *Pseudomonas putida*, and a high content of mobile genetic elements (7% of the genome)[27]. Thus, variability and flexibility in the interactions of *P. syringae* with plants is not surprising.

P. fluorescens strains are present in a wide range of terrestrial and aquatic environments, and although they are occasionally isolated as leaf endophytes, they are most commonly found as leaf epiphytes, soil bacteria, and root endophytes. *P. fluorescens* are generally regarded as harmless or beneficial bacteria, as the species includes PGPR and strains that suppress plant pathogens and induce plant disease resistance[116, 122, 157]. However, some *P. fluorescens* strains are associated with deleterious effects on plants and opportunistic infections of plants and other eukaryotes[75, 109, 122, 153]. *P. fluorescens* plant diseases are frequently associated with the production of cell wall degrading enzymes and surfactants such as viscosin, resulting in soft rot and wet rot symptoms[24, 122].

P. syringae and *P. fluorescens* share a common habitat in the form of plant surfaces, so it is not surprising that they have many traits in common, including traits involved in attachment and stress tolerance. Genomic and functional genomic analyses of these bacteria suggest that there may be a significant amount of horizontal gene transfer between the two species[27, 51]. Genomic rearrangements, gene transfer, plasmid transfer, and phase variation help both *P. syringae* and *P. fluorescens* to adapt to changing circumstances[12, 13, 28, 132, 144].

However, there do seem to be fundamental differences in the lifestyles and environmental niches of plant growth-promoting *P. fluorescens* and plant pathogenic *P. syringae. P. fluorescens* strains appear to be better adapted for survival in the complex, competitive world of the rhizosphere, but they are generally unable to reach the high endophytic population levels observed for *P. syringae* in susceptible hosts. Endophytic multiplication by *P. syringae* is highly host-specific, whereas many *P. fluorescens* strains are able to colonize the roots and leaves of a wide range of hosts, as well as interacting with other soil-borne eukaryotes such as fungi, nematodes, and arthropods. This suggests that the two species have distinct strategies for success.

The typical *P. fluorescens* is a generalist, able to thrive moderately well under a wide range of conditions. Its large genome encodes a flexible regulatory network and a wide range of genes for nutrient acquisition, stress tolerance, antibiotic synthesis, attachment, and biofilm formation[17, 51, 122]. *P. syringae* strains appear to be more specialized for an epiphytic and endophytic lifestyle, with specialization probably driven by selection for evasion or suppression of host recognition, with the payoff of an exclusive niche and a population boom in susceptible host plants. Specialization in *P. syringae* has also been accompanied by diversification in the form of a wide variety of virulence factors, and modifications to extracellular components such as flagellins, which can be recognized by host defense systems[11, 45, 72, 146, 147]. Increased specialization is often associated with the loss of certain metabolic functions, and the genome of *P. syringae* pv. tomato lacks metabolic genes such as arginine dihydrolase and subunits of the aa_3-type cytochrome oxidase[27] that are present in *P. fluorescens*. Although many *P. syringae* are seed-borne pathogens, they fare poorly in the rhizosphere compared to *P. fluorescens* strains. This may be due to an inability to compete for nutrients or survive antagonism by other soil organisms, as they will colonize sterile roots and pasteurized soil (G. Preston, unpublished; C. Bull, 1987, M.S. Thesis).

It is important to emphasize that this overview of the differing interactions of *P. syringae* and *P. fluorescens* with plants is a generalization, and that individual strains may defy this categorization, particularly given the opportunities available to these bacteria to exchange genetic material. Many epiphytic *P. syringae* strains are not demonstrably pathogenic, some

P. fluorescens strains express "pathogenicity" factors commonly associated with *P. syringae* or other pathogenic *Pseudomonas* spp. One clear example of this is the quintessential *P. syringae* pathogenicity mechanism—the Hrp TTSS pathway.

2. THE HORIZONTAL JOURNEY OF TTSS GENES INTO *PSEUDOMONAS* SPP.

Regions in bacterial genomes that have been acquired by horizontal gene transfer often show characteristic features such as altered GC content and flanking sequences that contain direct repeats, insertion sequence (IS) elements, tRNA genes, and genes for transposases[59]. Analyses of the sequences flanking the TTSS gene clusters in *P. syringae* and *P. fluorescens* tell us something of the history of the TTSS gene cluster, particularly in the case of *P. syringae*. Figure 1A outlines the genetic structure of regions flanking the TTSS gene clusters in *P. syringae* pv. tomato DC3000 and *P. fluorescens* SBW25, along with equivalent regions from *P. aeruginosa* PAO1 and *P. putida* KT2440. As elaborated further below, the exchangeable effector loci (EELs) of several *P. syringae* strains have been extensively characterized and shown to contain a variety of TTSS effector genes, pseudogenes, and mobile genetic elements. The region flanking the EEL is conserved in all the other *Pseudomonas* spp., as is the region on the RspR side of *P. fluorescens* SBW25.

Genomic data suggest that the conserved genes immediately flanking the clusters with *queA, ggt, gst, mcp*, and *gtp*, may be adjacent to, or act as, "hotspots" of recombination in *Pseudomonas* genomes, as the sequences located on one or both sides of these genes are highly divergent. In the case of *queA* and *gtp*, a high frequency of recombination may be linked to the presence of a tRNA gene. Although *P. syringae* pv. tomato does not encode a Met-tRNA gene immediately adjacent to *gtp*, there is Met-tRNA 52 kb upstream of *gtp*, separated by a region that is rich in integrases, transposases, plasmid genes, methylases, and glycosyl transferases. It is possible that the region adjacent to *gtp* in *P. syringae* pv. tomato may be an integrated region. Many *Pseudomonas* genomes contain multiple gamma-glutamyl transpeptidase (*ggt*), glutathione-S-transferase (*gst*), and methyl-accepting carrier (*mcp*) family genes. *mcp* genes are located adjacent to a wide range of virulence and plant colonization genes in *P. syringae* and *P. fluorescens*, including those for biosynthesis of the phytotoxin syringomycin (*P. syringae* pv. syringae), for TTSSs (*P. fluorescens*), and TTSS effectors (e.g., $hopPsyA_{Pto}$ of *P. syringae* pv. tomato)[98, 123] (Preston, unpublished).

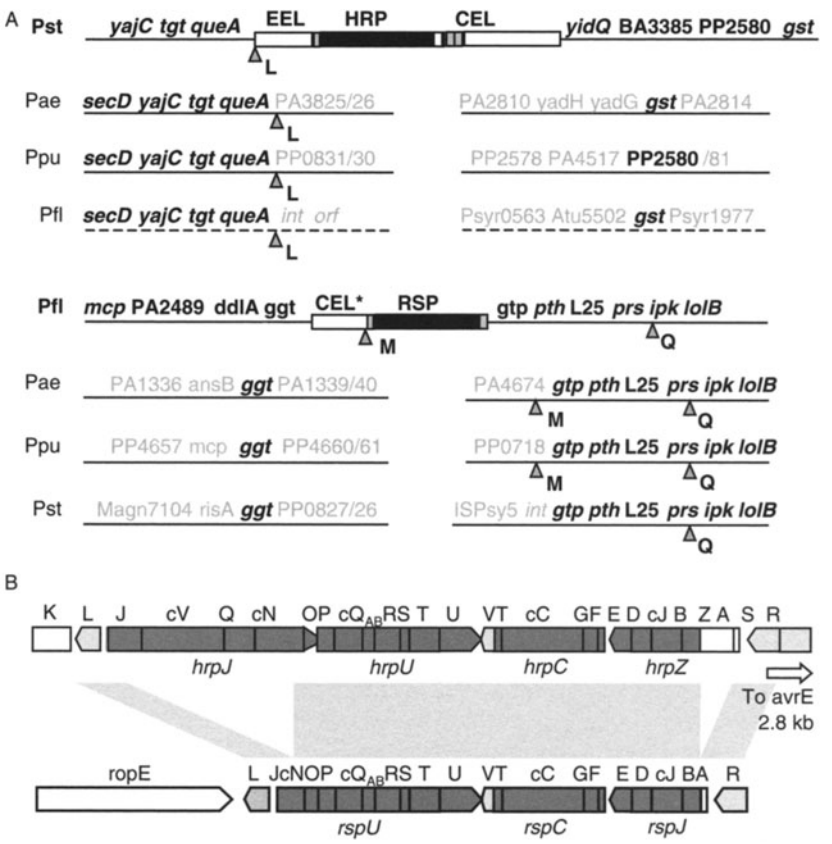
A
Pst yajC tgt queA EEL HRP CEL yidQ BA3385 PP2580 gst
L
Pae secD yajC tgt queA PA3825/26 PA2810 yadH yadG gst PA2814
L
Ppu secD yajC tgt queA PP0831/30 PP2578 PA4517 PP2580 /81
L
Pfl secD yajC tgt queA int orf Psyr0563 Atu5502 gst Psyr1977
L
Pfl mcp PA2489 ddlA ggt CEL* RSP gtp pth L25 prs ipk lolB
M Q
Pae PA1336 ansB ggt PA1339/40 PA4674 gtp pth L25 prs ipk lolB
M Q
Ppu PP4657 mcp ggt PP4660/61 PP0718 gtp pth L25 prs ipk lolB
M Q
Pst Magn7104 risA ggt PP0827/26 ISPsy5 int gtp pth L25 prs ipk lolB
Q
B
K L J cV Q cN OP cQAB RS T U VT cC GF E D cJ B Z A S R
hrpJ hrpU hrpC hrpZ
To avrE
2.8 kb
ropE L JcNOP cQAB RS T U VT cC GF E D cJ BA R
rspU rspC rspJ

Figure 1. TTSS gene clusters and genomic context in *P. syringae* and *P. fluorescens*. A. Genomic context of TTSS gene clusters in *P. syringae* pv. tomato (Pst) and *P. fluorescens* (Pfl) with corresponding regions of *P. aeruginosa* (Pae) and *P. putida* (Ppu) shown for comparison. Corresponding regions were selected on the basis of the region with highest similarity to a protein thought to be unrelated to TTSS function, located adjacent to the TTSS gene cluster. Triangles indicate presence of tRNA-like sequences, with letters (M, L, Q) indicating whether the putative tRNA is a methionine, leucine, or glutamine. EEL and CEL refers to the EEL and conserved effector locus as described in Alfano *et al.*[3] and in the text. Depictions of *P. fluorescens* SBW25 sequences are based on data generated by G. Preston and on unfinished genome sequence data produced by the *P. fluorescens* SBW25 Sequencing Group at the Sanger Institute. *P. fluorescens* SBW25 sequences can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/pf/. B. Organization of the core TTSS genes in *P. syringae* DC3000 and *P. fluorescens* SBW25. Conserved TTSS genes given a *hrc* or *rsc* designation are preceded by c, with the exception of the *hrcQ$_A$, hrcQ$_B$, hrcR, hrcS, hrcT, hrcU* region, which is shown as cQ$_{AB}$RSTU. All other genes are *hrp* or *rsp* genes with the exception of *ropE*. Genes encoding putative secreted proteins are shown as white boxes and regulators as pale gray boxes. The conserved proteins *avrE* and *ropE* are located on opposite sides of their respective TTSS clusters, and the location and distance of *avrE* relative to the *hrp* cluster is indicated on the right hand side of the figure. The predicted operon organization of the TTSS genes is indicated by box arrows, with the standard nomenclature for the TTSS operons given below the boxes (the putative *hrpJ* and *hrpU* operons in DC3000 may also be transcribed as a single unit).

2.1. Phylogeny of the TTSSs of *P. syringae* and *P. fluorescens*

Although phylogenomic evidence indicates that there is extensive variation and recombination in *P. syringae* effector loci, analysis of TTSS genes indicates that the conserved elements of the TTSS are evolutionarily stable within *P. syringae* pathovars[133]. However, the picture becomes more complicated when considering the distribution of TTSS genes across *Pseudomonas*. TTSS genes are present in at least four of the seven finished and unfinished *Pseudomonas* genome sequences currently available: *P. syringae* pv. tomato DC3000, *P. syringae* pv. syringae B728a, *P. fluorescens* SBW25, and *P. aeruginosa* PAO1, although the TTSS genes of *P. aeruginosa* PAO1 are not closely related to those of the plant-associated *Pseudomonas* (Figure 2). TTSS genes are not present in *P. putida* KT2440, *P. fluorescens* Pf0-1, and *P. fluorescens* Pf-5. All *Pseudomonas* genomes sequenced to date contain a substantial amount of strain specific genomic material, which probably reflects a high incidence of horizontal transfer and recombination for genes involved in certain niche-specific traits, including the TTSS and TTSS effectors, as discussed above. A recent study comparing the PAO1 genome to three additional draft-sequenced *P. aeruginosa* strains concluded that much of the genome, including the TTSS was highly conserved, but that there was approximately 10% of additional material in each genome relative to PAO1. Furthermore, sequence variation could be identified for extracellular factors subject to host-recognition, such as flagellin and O-antigen biosynthesis[139].

If we focus on closely related lineages of *Pseudomonas* with conserved features such as the ability to cause disease on specific hosts, as in pathogenic *P. syringae*, then we see the evolutionarily conservative picture of TTSS phylogeny described by Sawada *et al.* (1999). However, a recent analysis of TTSS distribution in a natural population of leaf-colonizing fluorescent *Pseudomonas* spp. shows that neither 16S rDNA data nor multilocus enzyme electrophoresis (MLEE) data are congruent with the distribution of TTSS genes in this population, as determined by PCR and hybridization, although TTSS genes are conserved in closely related lineages within this population[123] (Preston, Page and Rainey, unpublished). Current information on TTSS distribution and function is skewed toward characterization of pathogenic bacteria. Ongoing studies of TTSS genes in commensal and non-pathogenic bacteria from plants and animals are revealing a complex picture of TTSS distribution in bacterial populations, in which both TTSS genes and effectors are acquired, modified, and deleted[54] (M. Pallen, personal communication).

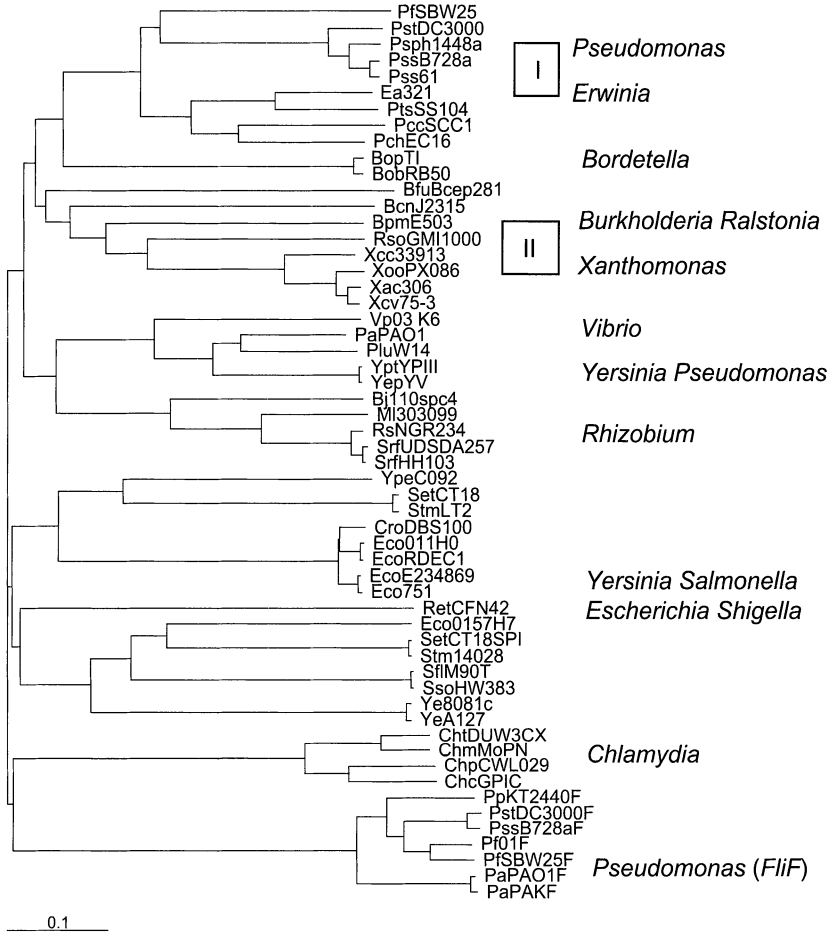

Figure 2. Phylogenetic tree of HrcJ homologs. Tree drawn using TreeView 1.6.6 (R. Page, Glasgow, UK), based on Clustal W[69] alignment of HrcJ-like sequences, including FliF sequences from *Pseudomonas*. Boxes are used to highlight the location of the group I and group II plant-associated TTSS genes. Abbreviations: *P. fluorescens* SBW25 (PfSBW25, AAK81934); *P. syringae* pv. phaseolicola 1448a (Psph1448a, AAF99294); *P. syringae* pv. tomato DC3000 (PstDC3000, NP_791211); *P. syringae* pv. syringae 61 (Pss61, AAC35801); *Erwinia amylovora* Ea321 (Ea321, AAB49174); *Pantoea stewartii* subsp. stewartii (PtsSS104, AAG01458); *Erwinia carotovora* subsp. carotovora SCC1 (PccSCC1); *Erwinia chrysanthemi* EC16 (PchEC16); *Bordetella pertussis* Tohama I (BopTI, CAC79572); *Bordetella bronchiseptica* RB50 (BobRB50, AAC38609); *P. aeruginosa* PAO1 (PaPAO1, AAG05112); *Burkholderia cenocepacia* J2315 (BcnJ2315, AAL59400); *Photorhabdus luminescens* W14 (PluW14, AA018027); *Vibrio parahaemolyticus* 03:K6 (Vp03:K6, BAC59953); *Bradyrhizobium japonicum* 110spc4 (Bj110spc4, AAG60796); *Yersinia pseudotuberculosis* YPIII (YpYPIII, AAA27651); *Yersinia enterocolitica* (pYV) (YepYV, A40049); *Sinorhizobium fredii* UDSDA257 (SrfUDSDA257, AAB17667); *Rhizobium* spp. NGR234 (RsNGR234, AAB91945); *Burkholderia pseudomallei* E503 (BpmE503,

2.2. Comparison of the TTSS Islands of *P. syringae* and *P. fluorescens*

Pseudomonas strains occupy a diverse range of ecological niches, which bring them into contact with a wide range of potential eukaryotic hosts. Variation in TTSS distribution can arise through three distinct processes: acquisition of a functional TTSS as a fitness or pathogenicity island; loss of function and deletion mutations in the TTSS in the absence of positive selection for TTSS function; and gain of function mutations or gene acquisitions in the presence of positive selection for TTSS function.

This model of TTSS acquisition, modification, and deletion is supported by sequence analysis of the TTSS clusters of *P. syringae* and *P. fluorescens*. The overall organization of the core components of the TTSS islands sequenced is very similar (Figure 1B), placing both clusters in the group I subgroup of plant pathogens as defined by gene similarities, operon structure, and regulatory factors[4] (Figure 2). However, there are three major differences between the TTSSs of *P. syringae* and *P. fluorescens*. First, *P. fluorescens* contains only one σ^{54} enhancer-binding protein (EBP), RspR. Second, the *P. fluorescens* cluster lacks a *hrpZ* or other harpin-like gene. Third, the *P. fluorescens* SBW25 cluster has a deletion within the *rspU* operon which means that this strain has only partial sequences for *hrpJ* and *hrcN* homologs, and lacks sequences homologous to *hrcV* and *hrpQ*. The TTSS island of *Erwinia amylovora* also has only one σ^{54}

Figure 2. Continued
AAK73236); *Mesorhizobium loti* MAFF303099 (Ml303099, NP_10683); *Sinorhizobium fredii* HH103 (SrfHH103, AAK60404); *Xanthomonas axonopodis* pv. citri 306 (Xac306, AAM35300); *Xanthomonas campestris* pv. campestris ATCC33913 (Xcc33913, AAM40531); *Xanthomonas campestris* pv. vesicatoria 75-3 (Xcv75-3, AAA27604); *Xanthomonas oryzae* pv. oryzae PX086 (XooPX086, AAF61284); *Ralstonia solanacearum* GMI1000 (RsoGMI1000, 2113360N); *Burkholderia fungorum* Bcep281 (BfuBcep281, NZ_AAAJ02000025); *Yersinia pestis* C092 (YpeC092, CAC89125); *Salmonella enterica* subsp. enterica serovar typhi CT18 (SetCT18II; CAD01956); *Salmonella typhimurium* LT2 (StmLT2ssa, CAA70532); *Rhizobium etli* CFN42 (RetCFN42, AAM54996); *Chlamydia trachomatis* D/UW-3/CX (ChtDUW3CX, AAC68161); *Chlamydia pneumoniae* CWL029 (ChpCWL029, AAD18965); *Chlamydia muridarum* (ChmMoPN, AE002160); *Chlamydia caviae* GPIC (ChcGPIC, AAP05674); *S. enterica* subsp. enterica serovar typhi CT18 (SetCT18I, CAD05978); *S. typhimurium* ATCC14028 (Stm14028, AAB60191); *Y. enterocolitica* 8081c (Ye8081c, AAF82342); *Shigella flexneri* M90T (SflM90T, AAA26532); *Shigella sonnei* HW383 (SsoHW383, BAA09148); *E. coli* O157:H7 (Eco0157H7, AAG57972); *Y. enterocolitica* A127 (YeA127, AAK84104); *E. coli* O11:H0 (Eco011H0, AAL30751); *Citrobacter rodentium* DBS100 (CroDBS100, AAL06364); *E. coli* RDEC-1 (EcoRDEC1, AAK26710); *E. coli* E2348/69 (Eco234869, AAC38379); *E. coli* 75.1 (Eco751, AAL58461); *P. putida* KT2440 FliF (PpKT2440F, AAN69947); *P. syringae* pv. tomato DC3000 FliF (PstDC3000F, AAO55476); *P. syringae* pv. syringae B728a FliF (PssB728aF, ZP_00127273); *P. aeruginosa* PAO1 FliF (PaPAO1F, AAG04490); *P. aeruginosa* PAK FliF (PaPAKF, AAB06801); *P. fluorescens* Pf0–1 FliF (Pf01F, ZP_00084952); *P. fluorescens* SBW25 FliF (PfSBW25, unfinished genome).

EBP gene, which suggests that duplication of an ancestral *hrpR/hrpS* homolog may have occurred within the *P. syringae* lineage. The availability of the draft *P. fluorescens* SBW25 genome allows us to confirm that this strain lacks homologs of the *hrpJVQN* region and *hrpZ*, and that the closest homologs to the missing conserved *hrc* genes are predicted to be involved in flagellar biosynthesis (Preston, unpublished). The deletion within the *rspU* operon and the lack of *hrpZ* may explain why this strain never elicits more than a weak HR, even when expressing heterologous elicitors from *P. syringae*. However, it is important to note that the *P. fluorescens* SBW25 TTSS may be atypical in this regard. Hybridization and sequence analyses of related TTSSs in other *P. fluorescens* strains show that at least some strains do possess intact copies of *hrcV* and other genes that are absent in *P. fluorescens* SBW25 (Preston, unpublished).

Pseudomonas fluorescens SBW25 does not possess an extensive array of TTSS-linked genes flanking the TTSS. Only one effector gene is conserved in the Rsp "island" relative to *P. syringae*, the *ropE* gene, which is homologous to *avrE* from *P. syringae* and *dspA/dspE* from *E. amylovora* (Preston *et al.*, 2001). *ropE* is located on the *rspL* side of the cluster rather than the *hrpRS* side. Hybridization and sequence analyses indicate that *ropE* is conserved in a wide range of TTSS-expressing *P. fluorescens* strains, and probably has a conserved function exploited by both pathogens and PGPR.

2.3. Comparison of the Exchangeable Effector Loci (EELs) of Various *P. syringae* Pathovars

A striking feature of the TTSS island of *P. syringae* is that it has a tripartite structure. A central *hrp/hrc* region is flanked by an EEL that can differ among strains of the same pathovar and by a conserved effector locus that appears similar in the divergent pathovars syringae and tomato[3]. TTSS effector genes are distributed, often in clusters associated with mobile genetic elements, around the chromosome of *P. syringae* pv. tomato DC3000, and they are also common on plasmids in various *P. syringae* strains[27, 158]. The EEL provides many clues to the evolution of the *P. syringae* TTSS/effector system.

The EEL was initially defined by sequencing the region between *hrpK* and *queA* in *P. syringae* pv. syringae 61, *P. syringae* pv. syringae B728a, and *P. syringae* pv. tomato DC3000[3]. Strikingly, 3 nt downstream of the *hrpK* stop codon, the sequences are completely divergent in these three strains until conserved $tRNA^{leu}$-*queA-tgt* sequences are encountered, as described above. EELs contain genes predicting effectors and mobile genetic elements, and they have an unusually low G+C content[3]. An interesting feature observed in the

prototypical EELs of B728a and DC3000 was the presence of effector genes in the *hrpK* transcriptional unit, which is activated by HrpL (discussed below). Thus, recombination at this locus can result in recruitment of new effector genes into the Hrp regulon.

Conserved *queA* sequences were used to PCR-amplify part of the *P. syringae* pv. syringae 61 EEL, and the similar use of both *queA* and *hrpK* or *hrpL* sequences recently enabled amplification of more than 20 additional EELs from various *P. syringae* pathovars[32, 41]. The sequences of these EELs provided several further insights into this region and *P. syringae* evolution. First, the region between *hrpK* and $tRNA^{leu}$ in every strain examined contains at least one effector gene (or effector pseudogene) and, in many cases, mobile genetic elements. The number of ORFs in known EELs ranges from six (including three encoding effectors that have been demonstrated to be functional based on secretion and avirulence activity) to a single pseudogene, as observed in *P. syringae* pv. syringae B728a and *P. syringae* pv. mori PDDCC 4331, respectively. A second important finding is that several of the effector genes in this region are widespread among *P. syringae* strains (although located outside of the EEL in some strains). Homologs of *avrPphE* and *hopPsyA* are particularly common, although their orientation in the EEL can vary, and in some cases they are represented by pseudogenes. A third notable finding is that the phenotypes of EEL deletion mutants do not suggest any universal function in pathogenesis for this region: A *P. syringae* pv. tomato DC3000 mutant is slightly reduced in its growth *in planta*, whereas a *P. syringae* pv. syringae B728a is strongly reduced in virulence[3, 41]. Finally, an analysis of the genomic locations, synonymous mutations, and phylogenetic trees of *avrPphE* and *hrpK* suggests that *avrPphE* was acquired by *P. syringae* after *hrpK* and the rest of the Hrp system but before the divergence of modern host-specific pathovars[41]. Consistent with this concept, there is no correlation between the structure of EELs and the host range of *P. syringae* strains.

Mosaic pathogenicity islands have been observed in other pathogens[67], and the presence of mobile genetic elements and sequences such as effector genes, which are frequently associated with plasmids and other mobile DNA, can promote ongoing cycles of recombination in regions such as the Hrp pathogenicity island EEL of *P. syringae*. In this regard, it is interesting to note that, whereas *avrPphE* is prevalent in the EELs of the *P. syringae* strains studied, it is carried on a plasmid in *P. syringae* pv. tomato DC3000, and it is duplicated on both a plasmid and the chromosome in *P. syringae* pv. maculicola M6. Furthermore, in M6, the *avrPphE* locus is a site of integration for a plasmid that carries at least one other effector, which also becomes integrated into the chromosome by this process[129].

3. TTSS REGULATION: ORGANIZING THE JOURNEY OF EFFECTOR PROTEINS INTO HOST CELLS

One important route to understanding the role of TTSS genes in *Pseudomonas* is to know when and where they are expressed. *P. syringae* rapidly up-regulates *hrp* gene expression after inoculation into plant tissues. The minimal requirements for *hrp* gene expression in *P. syringae* have been known since the 1980s. An apoplast-mimicking, acidic minimal medium that contains fructose as a carbon source induces the expression of *hrp* genes in many *P. syringae* pathovars[79, 126, 164]. Tricarboxylic cycle intermediates such as citrate, succinate, and L-glutamate effectively suppress *hrp* induction, indicating that TTSS expression is subject to catabolite repression. Osmolarity has also been shown to affect TTSS expression, which may reflect the effect of osmotic stress on DNA bending and supercoiling in promoter regions[126].

In contrast to *P. syringae*, the *rsp* genes of *P. fluorescens* are not induced in Hrp minimal medium, and the signals leading to upregulation of *rsp* gene expression in *P. fluorescens* remain unclear, although *rsp* genes are induced in the rhizosphere and expressed at a low level *in vitro*. *rsp* gene expression may be subject to negative regulation *in vitro* and may require rhizosphere-specific signals for activation during plant colonization. Plant-specific signals have been shown to be involved in upregulation of TTSS expression in *Ralstonia* and *Rhizobium*, and some *Pseudomonas hrp* genes are significantly up-regulated *in planta*, compared to inducing medium[101, 102, 126, 164]. Additional factors known to affect TTSS expression in mammalian pathogens include the status of TTSS assembly and function, bacterial growth phase, cell division, Dam methylation, and flagella biosynthesis[14, 83, 99], but none of these factors has been extensively studied with respect to TTSS expression in *P. syringae* or *P. fluorescens*.

3.1. The TTSS Regulatory Hierarchy

hrp and *rsp* transcription are regulated by positive regulatory proteins encoded within the TTSS gene cluster (Figure 1). An outline of the *hrp* regulatory cascade is shown in Figure 3. The σ^{54} EBPs HrpR and HrpS activate transcription of HrpL from an RpoN (σ^{54}) dependent promoter. RpoN has been shown to be required for TTSS expression and virulence in *P. syringae* pv. *maculicola*[65, 66]. HrpL activates transcription of all the other components of the Hrp secretion apparatus, and of many, if not all, Hrp-secreted effectors in *P. syringae*. A similar regulatory hierarchy operates in *P. fluorescens* and *Erwinia* spp., in which a single σ^{54}-activating EBP, RspR or HrpS respectively, activates transcription of RspL or HrpL[123, 161] (Jackson, Preston, Bertrand, and Rainey, unpublished).

The mechanisms underlying environmental regulation of TTSS expression in *Pseudomonas* remain unclear. HrpR and HrpS function optimally as a heteromer, although some strains show a low level of *hrpL* activation in response to HrpS alone[78]. HrpR, HrpS, and RspR are unusual RpoN-activating proteins in that they consist of only a DNA-binding domain and an RpoN-activating domain, and lack any additional modulating domain to transduce environmental signals[78]. Environmental sensing of nutrient status and pH in *P. syringae* must be mediated through transcriptional regulation of *hrpR, hrpS*, and *hrpL*, by environmentally induced conformational changes in these regulatory proteins, or through interactions with other environmentally responsive factors, including RpoN itself and factors that alter DNA bending and supercoiling. Post-transcriptional regulation mechanisms could act by altering mRNA stability, DNA-binding, HrpR/HrpS multimerization, or RpoN activation by HrpR and HrpS.

In *E. amylovora*, transcriptional regulation of the TTSS is controlled by a two-component regulatory system encoded by HrpX and HrpY[161]. However, current evidence suggests that transcriptional regulation of *hrpR* and *hrpS* may not play an important role in regulating TTSS expression in *P. syringae*. Two factors shown to have an impact on *hrp* expression are degradation of HrpR by Lon protease in non-inducing conditions, and negative regulation of *hrpL* expression by HrpV, possibly through a direct interaction between HrpV and HrpR or HrpS (Figure 3)[25, 120]. Intriguingly, the SPI1 TTSS of *Salmonella enterica* serovar *typhimurium* is also regulated by Lon protease, even though it is activated by different regulatory cascades[150, 151]. Lon protease is an ATP-dependent protease that has a similar ATPase domain to σ^{54}-activating proteins such as HrpR and HrpS, although it is not known whether this similarity plays any role in targeting Lon protease to attack HrpR under specific environmental conditions.

Before leaving the subject of HrpR and HrpS, it is important to note that although the general model of *hrp* regulation in Figure 3 fits the observed behavior of many *P. syringae* strains, some details may vary. Several studies have shown that the *hrp* regulatory system of *P. syringae* pv. phaseolicola may have unique features not observed in *P. syringae*, including regulation of *hrpS* expression by *hrpR*[56, 126, 134].

3.2. The *hrp* Box

Comparative analyses of promoters activated by HrpL and RspL have revealed a conserved motif known as the *hrp* box, which was originally defined as GGAACC 15/16 bp CCAC[80, 137, 163]. This motif has been refined through comparative analyses of many HrpL-regulated promoters to the sequence shown in Figure 3[49]. HrpL and RspL belong to the extracytoplasmic

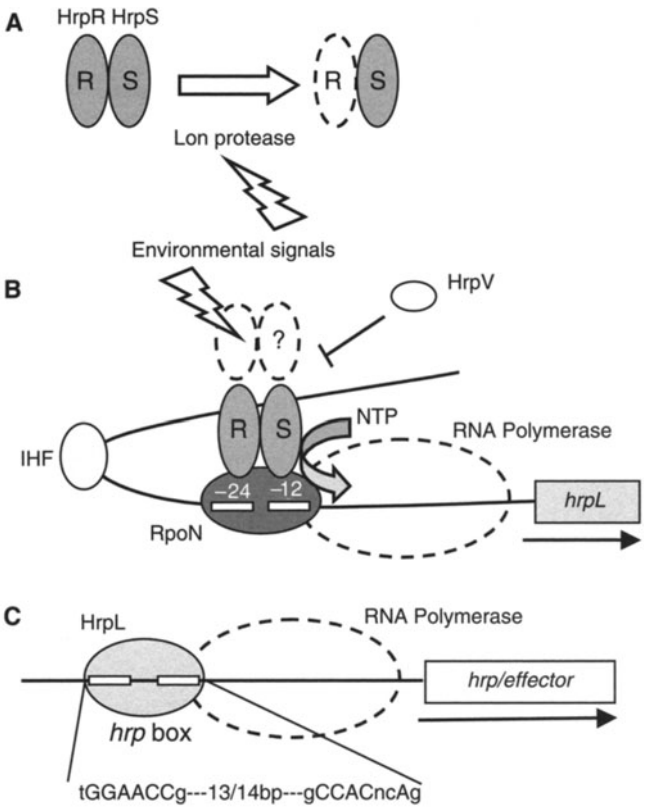

Figure 3. TTSS regulation in *P. syringae*. Positive regulatory proteins HrpR and HrpS (R, S) interact to form a multimeric complex that activates transcription of the *hrp*-specific sigma factor HrpL (panels A and B). HrpL in turn activates transcription of operons encoding the Hrp secretion machinery and Hrp-secreted effectors (panel C). A. HrpR is subject to degradation by Lon protease in *P. syringae*, providing one level of environmental regulation. B. HrpR and HrpS interact with the RpoN (σ^{54})-dependent promoter of *hrpL* to activate *hrpL* transcription. The presence of a putative integration host factor (IHF)-binding site upstream of the RpoN-binding motif indicates that IHF-mediated DNA-bending facilitates the formation of an active complex. Positive regulation by HrpR and HrpS is subject to repression by HrpV, and induction may be influenced by environmental factors acting directly or indirectly on (i) RpoN (ii) DNA bending and supercoiling and (iii) HrpR and HrpS.

function (ECF) family of sigma factors, and this consensus pattern fits the $-35,-10$ spacing observed for other sigma factors in this family[104]. However, although deletion analyses suggest that the *hrp* box is involved in promoter activity[137], no studies have conclusively linked the specific residues of the *hrp* box motif to HrpL binding, and the promoters of several HrpL-induced genes

deviate from this consensus sequence[49]. The presence of a *hrp* box has been used to identify putative effectors in the finished and unfinished genomes of *P. syringae* and *P. fluorescens*. Several of these putative effectors have subsequently been confirmed to be HrpL-regulated and secreted[49, 58, 117, 169].

3.3. Cross-Talk between Regulatory Pathways

RpoN also regulates a wide range of other phenotypic traits in *Pseudomonas*, including motility, nutrient acquisition, nitrogen metabolism, virulence, and biofilm formation[65, 66, 142]. An important question for researchers studying *hrp* regulation and function is how *hrp* expression is integrated into the complete picture of bacterial activities in the natural environment and how bacteria coordinate the expression of diverse traits. Many animal pathogens such as *P. aeruginosa* alter or down-regulate functions such as motility in the course of cell-contact-dependent interactions and biofilm formation and may tailor expression of virulence factors to specific stages in the infection process[61, 62]. It is therefore interesting to note that electron microscopy studies of Hrp secretion structures *in vitro* and *in vivo* have frequently shown flagella and Hrp pili present in the same preparation[26, 76], and that absence of the flagella TTSS system does not inhibit the HR[76]. However, regulatory mutants of *P. syringae* pv. tomato DC3000 that lack *hrpS* and *hrpV* produce significantly higher levels of the phytotoxin coronatine, indicating that there is significant feedback and cross-talk within the RpoN-dependent regulatory cascades governing both *hrp* and coronatine expression[115]. Further evidence of coordinated expression of *hrp* genes and other plant colonization traits comes from the screens for HrpL-regulated genes, which show that the coronatine biosynthetic genes *cfa1* and *cfa6*, and the auxin biosynthetic gene IaaL are regulated by HrpL. The same study also identified *hrp* boxes upstream of the coronatine regulatory gene CorR and a homolog of the syringomycin toxin biosynthetic gene *syrE*[49].

4. ASSEMBLING THE TTSS: FLAGELLAR PARALLELS

HrpL and RspL induce the expression of at least four polycistronic operons that encode the proteins comprising the TTSS. Nine of these proteins are highly conserved in most functional TTSSs and represent the core components of the TTSS needed to export proteins across the inner and outer bacterial membranes[18, 77]. With the exception of HrcC, which belongs to the secretin family of outer membrane porins, these core Hrc proteins share sequence similarities with flagellar assembly components[77]. The flagellar export system

is probably an evolutionary ancestor or relative of the TTSS, and in *Yersinia*, has even been shown to export TTSS effector proteins[166, 167]. Many of the conserved features of the structure and function of TTSSs have been extensively discussed in recent reviews[1, 16, 64, 77]. According to Blocker *et al.*[16], TTSSs are generally characterized by (a) host-contact mediated induction; (b) energy requirement for protein secretion and translocation; (c) secretion-regulated expression of secreted proteins; and (d) dedicated chaperones for some secreted proteins. TTSS-secreted proteins lack cleaved signal sequences and periplasmic secretion intermediates. We discussed the evidence for host-dependent induction of TTSS transcription in the preceding section. Here we offer a brief overview of the TTSS machinery with a focus on those features that are particularly noteworthy regarding the TTSSs of *P. syringae* and *P. fluorescens* in relation to this generic model of TTSS operation, as summarized in Table 1.

4.1. Conserved Core of the TTSS in the Bacterial Envelope

The TTSS of several mammalian pathogens has been shown to exist as a stable structure that physically resembles the flagellar basal body. This "needle complex" consists of a short (<100 nm long, 8 nm diameter) surface appendage called the needle, inserted in a 30 nm diameter complex that traverses both bacterial membranes and the peptidoglycan cell wall[15, 90, 135, 152]. Some mammalian pathogens also produce additional extracellular appendages[135]. The needle complex has not been isolated from *P. syringae* or *P. fluorescens*, but the TTSS of *P. syringae* has been shown to elaborate a filamentous surface appendage known as the Hrp pilus, which is approximately the same diameter, but which can be several micrometers long, as discussed below[82, 130].

At the heart of the export apparatus is a triplet of three highly conserved membrane proteins FliP/Q/R (cf. HrcR/S/T). Combinations similar to this triplet can be found in the Sec system (Sec F/E/Y) and in the F_0F_1-ATPase (ATP5/8/6), which suggests that this triplet is an ancient structure or protochannel for membrane channeling[1]. The membrane proteins FlhA and FlhB (HrcV and HrcU) are also conserved in most TTSSs, including the TTSS of *P. syringae* and some *P. fluorescens* strains, although RscV (= HrcV) is absent in *P. fluorescens* SBW25. FliY (FliN + FliM) is present in the TTSSs of *P. syringae* and *P. fluorescens* as two proteins, $HrcQ_A$ and $HrcQ_B$, rather than as a single protein as in most other TTSSs. Blocker *et al.*[16] propose that the powerhouse for TTSS secretion is a basally located ATPase, with homology to FliI (HrcN). They propose that the ATPase docks as a homohexamer at the inner membrane base of the needle complex, forming a central channel through which proteins are exported. If this model is accurate, it suggests that the normal power supply for protein unfolding and TTSS transport across the

Table 1. Functions of *P. syringae* and *P. fluorescens* TTSS components.

Gene	Flagellar homolog	Cellular location	Features	Predicted function
Core channel components				
HrcR	FliP	IM	N-terminal signal peptide, 3 TM domains, FliP family	Membrane channel
HrcS	FliQ	IM	N-terminal signal peptide, 1 TM domain, FliQ family	Membrane channel
HrcT	FliR	IM	3 TM domains, FliR family	Membrane channel
HrcU	FlhB	IM	4 TM domains, FlhB family	Membrane channel
HrcV	FlhA	IM	6 TM domains, FlhA family	Membrane channel
HrcN	FliI	C (peripheral)	ATP synthase beta-barrel and nucleotide binding domain, FliI family	Energy source for protein unfolding/transport
Basal components				
HrcJ	FliF	IM/OM	N-terminal signal peptide, lipoprotein? 1 TM domain, FliF family	MS-ring structure in basal complex
$HrcQ_A$	FliM	IM/C	FliM family	Basal complex/channel
$HrcQ_B$	FliN	IM/C	FliN family	Basal complex/channel
HrpQ	FliG?	IM	1 TM domain	Basal complex
Outer ring components (secretin)				
HrcC		OM/PP	N-terminal signal peptide, GspD family	Outer membrane porin
HrpT?		OM/IM	N-terminal signal peptide, lipoprotein?	Chaperone/partner for HrcC?
Pilus components				
HrpA		PP/EX	TTSS-secreted	Pilin component of Hrp pilus
Accessory proteins				
HrpZ		C/EX	TTSS-secreted Gly-rich protein	Translocation accessory protein?
HrpW		C/EX	TTSS-secreted Gly-rich protein with pectate binding domain	Translocation accessory protein?

Table 1. Continued

Gene	Flagellar homolog	Cellular location	Features	Predicted function
Other/unknown function				
HrpB	FlgD?	C/IM?	Similar to YscI	Basal complex?
HrpD		?	Similar to YscK?	Basal complex?
HrpE	FliM/H?	IM?	Similar to YscL, possible N-terminal signal peptide	Basal complex?
HrpO		?		Basal complex/exported protein?
HrpP		?		Basal complex/exported protein?
HrpJ		?		Basal complex/exported protein?
HrpF		?		Basal complex/channel?
HrpG		?		Basal complex/channel?
HrpM		IM	6 TM domains, glycosyltransferase family, II	Periplasmic glucan synthase

inner membrane is unplugged or attenuated in *P. fluorescens* SBW25, which encodes a truncated copy of the FliI-like ATPase RscN.

Export across the outer membrane is via a porin, HrcC, which belongs to the PulD family of outer membrane secretins involved in transport of macromolecules across membranes. This family includes proteins involved in two terminal branches of the general secretory pathway (GSP), type II secretion and type IV pilus biogenesis, and in competence and phage assembly[77]. The HrcC homolog YscC has been shown to assemble into 20 nm diameter, multimeric ring-like structures with a central pore[89]. Secretion by the type II secretion pathway is a two-step process with a periplasmic intermediate. Transport across the inner membrane requires ATPase activity and ΔP, the electrochemical proton gradient across the membrane. Transport across the outer membrane by secretins such as PulD in *Klebsiella* has been shown to depend on proton motive force rather than ATPase activity[48, 119], while transport of a GSP-dependent endonuclease across the outer membrane of *Serratia marcescens* and of aerolysin across the outer membrane of *Aeromonas salmonicida* is strongly affected by the pH gradient across the outer membrane[143].

In contrast to the GSP, TTSS secretion does not normally involve a periplasmic intermediate, although HrcC mutants accumulate the secreted protein HrpZ in the periplasm[34], and *Salmonella* flagellar proteins can be exported to the periplasm of appropriate mutants[103]. It is not clear whether TTSS transport across the inner and outer membranes requires either proton motive force or pH gradients as with protein secretion by the GSP, or whether the close association of inner and outer membrane components enables inner membrane motors to drive proteins across both membranes. The HrcC pore is probably closed when proteins are not in transit to avoid compromising the outer membrane, although the mechanism for opening and closing the pore is not known. HrcJ is homologous to FliF, a component of the MS-ring complex, and appears to bridge the inner and outer membrane components in *P. syringae*[39], where it may stabilize the channel between the two membranes or transduce energy or conformational signals between them.

4.2. Specialized Extracellular Components of the Hrp TTSS

Two extracellular components of the *P. syringae* TTSS have been identified so far: The *hrpA*-encoded Hrp pilus and harpins. The Hrp pilus has been shown to have an essential role in effector secretion and translocation[81, 92, 130, 160]. Harpins also are likely to have a role in effector translocation, but their actual function is less clear. HrpA and the harpins are far more abundantly secreted in culture by *P. syringae* than are effectors, which is consistent with their proposed function as secreted, rather than translocated, proteins[63, 130, 168].

The HrpA pilin subunit is encoded in the *hrp/hrc* gene cluster in an operon that also encodes the HrpZ harpin and HrcJ. Comparison of the *hrp* clusters of different *P. syringae* pathovars has revealed HrpA to be the most variable component of the system. For example, the predicted HrpA proteins of *P. syringae* pv. tomato DC3000 and *P. syringae* pv. syringae 61 share only 30% identity[40]. Despite this variability, the predicted secondary structures of the C-terminal regions of these proteins, beyond the first 50 amino acids, are α helices[88], and these appear important in pilus assembly because mutations affecting this region, in contrast to the N-terminal targeting region, disrupt pilus assembly[68, 149].

The Hrp pilus is a surface appendage that is 8 nm in diameter and may be several micrometers long[130]. The pilus elongates by adding new HrpA subunits at the distal end, which is mechanistically similar to the assembly of flagellin into flagellar filaments[92]. The Hrp pilus appears to be a conduit for delivering TTSS-secreted proteins to the cytoplasm of plant cells. Elegant experiments involving immuno-electron microscopy and pulse expression of AvrPto revealed that effectors travel through the pilus[81]. Since the internal diameter of the Hrp pilus is only 2 nm, it is likely that effectors pass through the pilus in an unfolded state[64].

The HrpZ harpin represents another class of proteins that have been shown to travel through the *P. syringae* Hrp pilus[92]. Furthermore, the presence of HrpZ along the length of pili that have been formed by *P. syringae* growing on electron microscopy grids indicates that harpins are extruded from the tips of elongating pili[26, 82]. This is one of many observations suggesting that the enigmatic harpins may have a role as extracellular components of the TTSS machinery.

Harpins are defined as proteins that travel the TTSS pathway, are glycine-rich, cysteine-lacking, and possess heat-stable HR elicitor activity when infiltrated into the leaves of tobacco and several other sensitive plants[4]. HrpZ is the first harpin found in *P. syringae*[63]. A second harpin is encoded by *hrpW* in the Hrp pathogenicity island conserved effector locus[33]. Analysis of the genomic sequence of *P. syringae* pv. tomato DC3000 has revealed two additional proteins, HopPtoP and HopPmaH$_{Pto}$, which possess all of the properties of harpins[35], including heat-stable activity as elicitors of the HR in tobacco (A.R. Ramos and A. Collmer, unpublished).

Although isolated *P. syringae* harpins have the ability to elicit the HR and other defense responses in plants[42, 63, 141, 165], the indistinguishable effects of harpins from different *P. syringae* pathovars on reciprocal host plants argues against any direct role in determining host specificity[121]. Furthermore, *P. syringae* mutants have so far provided little support for the role of the harpins in plant interactions. For example, mutations in *P. syringae* pv. tomato DC3000 affecting *hrpZ, hrpW*, or both genes have only a slight effect on plant interactions[2, 33], deleting

hrpZ from the *P. syringae* pv. syringae B728a genome has no effect on virulence under demanding field conditions[70], and multiple, virulent field strains of *P. syringae* pv. tabaci carry a natural deletion affecting much of *hrpZ*[148].

Harpin redundancy is one explanation for this apparent lack of a role, and this is supported by experiments involving *hrpZ* deletions in the cloned *P. syringae* pv. syringae 61 *hrp/hrc* cluster that is carried in pHIR11 and expresses only a single harpin[2]. Deletion of the *hrpZ* harpin reduces the ability of *P. fluorescens* carrying pHIR11 derivatives to elicit the HR[2, 118]. However, mutations affecting HopPsyA, the single effector encoded by pHIR11, completely abolish HR elicitation activity without affecting HrpZ secretion, which suggests that HrpZ plays a role in the delivery of HopPsyA, the actual elicitor of the HR.

Several other properties of harpins are consistent with a role in assisting the delivery of effectors, via the pilus, across the plant cell wall and plasma membrane. First, harpins have been reported to associate with walled plant cells but not with protoplasts[73]. Second, the *P. syringae* pv. tomato DC3000 HrpW and HopPmaH$_{Pto}$ harpins appear to be bifunctional proteins: the N-terminal half is harpin-like, and the C-terminal half shows *c.* 58% identity with pectic lyases[33]. Pectic enzymes degrade pectic polymers, which control the porosity and integrity of plant cell walls[9]. Although these harpins have no apparent enzymatic activity, HrpW has been shown to bind specifically to pectate[33]. The third indication for a role of harpins in effector translocation is found in the ability of the *P. syringae* pv. phaseolicola HrpZ to bind to the plant plasmamembrane and to form ion-conducting pores in artificial membranes[91]. However, we presently lack direct evidence that harpins are specifically involved in any aspect of effector translocation.

4.3. Components of the Hrp TTSS with Unknown Function

In addition to conserved flagellar homologs, the TTSS clusters of *P. syringae* and *P. fluorescens* encode several conserved proteins whose functions remain unknown. Most of these are important for TTSS function and are probably functionally equivalent to proteins characterized in other TTSSs but less conserved at a sequence level than "*hrc*" genes. As experimental knowledge of protein function and *in silico* predictions of protein structure become more sophisticated, it will probably be appropriate to reclassify most of these Hrp proteins as Hrc proteins. At present, the following similarities can be discerned:

- HrpB, HrpD, and HrpE (RspB, RspD, and RspE) may be equivalent to YscI, YscK, and YscL, and may encode needle components or chaperones[1, 121].

- HrpT (RspT) is a lipoprotein that may act as a chaperone for HrcC or otherwise be associated with the operation of the outer membrane pore. HrpT mutants are strongly attenuated in their ability to cause disease and elicit the HR[40].
- HrpQ (RspQ) is highly conserved in most group I, plant pathogen-associated TTSSs and may be functionally equivalent to proteins conserved in other TTSSs, including *Xanthomonas* (HrpD5), *Bordetella pertussis* (BscD), *P. aeruginosa* (PscD), *Ralstonia solanacearum* (HrpW), and *Salmonella typhimurium* (PrgH). PrgH and PrgK oligomerize into a ring-shaped structure identical in appearance and size to the base of the needle complex, and have been proposed to be two of the inner membrane structural components of the needle complex[86]. RspQ, like RscV, is absent in *P. fluorescens* SBW25, but present in several other strains of *P. fluorescens* (Preston, unpublished).
- HrpO and HrpP are probably equivalent to YscO and YscP. YscO is proposed to be a peripherally associated membrane protein involved in protein export, and there is evidence that YscO is itself secreted, although this has not yet been observed for functionally equivalent proteins in other bacteria[114]. YscP is also exported and surface exposed, and has been shown to have a role in substrate specificity, akin to the substrate switching observed during different stages of flagellar assembly[44].
- HrpF and HrpG are encoded upstream of HrcC and show no obvious similarity to previously characterized proteins. However, mutations in HrpF and HrpG reduce the ability of *P. syringae* to elicit the HR and to cause disease, so it seems likely that these proteins are also involved in some aspect of the operation of the TTSS[40].

One gene that has a Hrp phenotype, but which is not obviously associated with the TTSS is *hrpM*[110]. *hrpM* encodes a protein with homology to the osmoregulated periplasmic glucan synthase gene MdoH[97]. Interestingly, mutations of OpgH, the *Erwinia chrysanthemi* equivalent of MdoH, are also greatly attenuated in virulence and have a pleiotropic phenotype that includes alterations in motility, exoenzyme synthesis, exopolysaccharide synthesis, and envelope structure[112]. Mutants of the periplasmic glucan synthases of *E. coli* and *Agrobacterium tumefaciens* also display defects in flagellar biosynthesis and assembly, and in the structural stability of the type IV secretion apparatus[20, 29, 47]. It would not be surprising to find that *hrpM* mutations are affecting TTSS assembly and function in a similar fashion at a transcriptional or post-translational level.

5. TTSS PROTEIN TRAFFIC CONTROL: TARGETING SIGNALS AND CHAPERONES

One essential function of the TTSS is in recognizing the subset of proteins or nascent proteins in the bacterium that are to be targeted to the pathway. This process involves targeting signals that are associated with TTSS effectors and, in some cases, Shc (*s*pecific *H*op *c*haperone) proteins that may serve individual effectors. Although the process by which effector proteins are targeted to the TTSS in various pathogens is still poorly understood, some aspects of this puzzling phenomenon are coming into clearer focus and, as described below, we have learned enough to successfully predict at least some of the proteins that are capable of traveling the pathway.

5.1. Targeting Signals in TTSS Substrates

A noteworthy feature of the TTSS is that the pathway is capable of secreting heterologously expressed effectors derived from other TTSS pathogens. This phenomenon was first observed with the reciprocal secretion of TTSS substrates by *Yersinia, Shigella*, and *Salmonella* spp.[131], and it implies that the targeting signals associated with these proteins are functionally similar. Subsequently, it was demonstrated that the TTSS of *E. chrysanthemi* (a pathogen that causes soft rots in many plants) and *Yersinia enterocolitica* can secrete *P. syringae* effectors[5, 60]. The use of a universal targeting signal for TTSS substrates is consistent with the observation that some effector genes are present in multiple genera of plant pathogens. For example, homologs of *avrE*, an effector gene found in the *P. syringae* conserved effector locus, are also found in *Erwinia amylovora, Erwinia carotovora, Erwinia herbicola* pv. gypsophilae, and *Pantoea stewartii* subsp. stewartii[10, 19, 52, 106]. Furthermore, *P. syringae avrE* can at least partially restore virulence to an *E. amylovora dspE* (*avrE* homolog) mutant, and *dspE* confers an avirulence phenotype to *P. syringae* pv. glycinea[19].

Universal targeting signals suggest that components controlling entry into the TTSS pathway are similarly universal. Supporting this concept, the *hrp*/*hrc* cluster of *P. syringae* pv. tomato can be replaced with that of the bean pathogen *P. syringae* pv. syringae 61 without loss of host-specificity or pathogenicity on tomato[50]. All of these observations suggest that the many pathovars and races of *P. syringae* possesses Hrp systems that are functionally equivalent in their basic ability to deliver a dynamic collection of effectors to a wide range of potential host plants.

Pioneering work on the Yop effectors of *Yersinia* spp. has established that key targeting information resides in the first 15 codons and/or amino acids. In addition, some effectors have chaperone-interaction domains that may extend from amino-acid 15 to amino-acid 150. Thus, effectors have a

general multidomain structure, with the N-terminal regions carrying information for targeting to the TTSS (and in some cases to specific host organelles) and the C-terminal regions directing effector activity in host cells. As explained below and depicted in Figure 4, *P. syringae* effectors also have this structure, which has enabled the development of new tools for exploring the TTSS pathway and the nature of the targeting signals.

Deletion mutations demonstrated that essential targeting information is located in the first 10 and 15 amino acids of the *P. syringae* AvrPto and AvrB proteins, respectively[5]. Analysis of the fate of AvrRpt2 in Arabidopsis during infection with *P. syringae* or when *avrRpt2* is expressed as a transgene in the plant suggest that the N-terminus of the protein is processed within plant cells, and the processed C-terminal portion of the protein is recognized by the cognate RPS2 resistance protein to produce an avirulence phenotype[108]. The avirulence activity of AvrRpt2 recently gained even more significance with

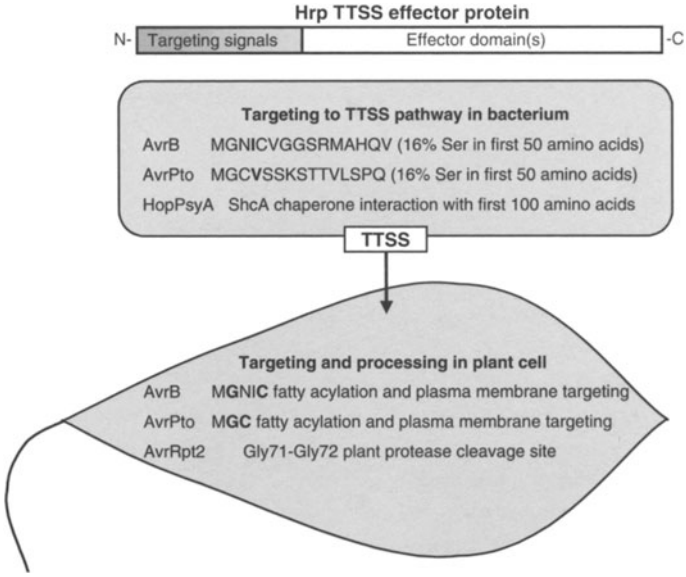
Hrp TTSS effector protein
N-
Targeting signals
Effector domain(s)
-C
Targeting to TTSS pathway in bacterium
AvrB MGNICVGGSRMAHQV (16% Ser in first 50 amino acids)
AvrPto MGCVSSKSTTVLSPQ (16% Ser in first 50 amino acids)
HopPsyA ShcA chaperone interaction with first 100 amino acids
TTSS
Targeting and processing in plant cell
AvrB MGNIC fatty acylation and plasma membrane targeting
AvrPto MGC fatty acylation and plasma membrane targeting
AvrRpt2 Gly71-Gly72 plant protease cleavage site

Figure 4. Signals in *P. syringae* Hrp effector proteins involved in targeting to the TTSS pathway and then to subcellular locations within plant cells. Effector proteins have a multi-domain structure. The N-terminal region contains TTSS targeting information, with the first 10–15 codons being essential for secretion or translocation through the pathway. The N-terminal region contains characteristic patterns, for example, an aliphatic amino acid in positions 3 or 4, and is typically rich in Ser. Some effectors, such as HopPsyA also have a chaperone-interaction domain, which may extend from positions 16 to 100 or more. Within plant cells, the N-terminal regions of AvrB, AvrPto, and some other effectors can be fatty-acylated at Gly and Cys residues, resulting in localization to the plant plasma membrane. Other effectors, such as AvrRpt2, may be proteolytically processed *in planta*.

reports that this activity is dependent upon the ability of the effector to cause the degradation of the RIN4 susceptibility target[7, 100]. Thus, the C-terminal portion of AvrRpt2, sans the targeting region, appears sufficient for both virulence and avirulence activities of the protein.

Importantly, fusion of the biologically active C-terminal portion of AvrRpt2 to the N-terminal regions of the *Xanthomonas campestris* pv. vesicatoria AvrBs2 or *P. syringae* pv. maculicola AvrRpm1 effectors results in translocation of the hybrid proteins into plant cells, as indicated by elicitation of the HR in infected Arabidopsis plants in a TTSS- and RPS2-dependent manner[57, 107]. This use of the C-terminal effector domain of AvrRpt2 provides a means to determine whether a candidate effector carries TTSS targeting information in its N-terminal regions, and this use was extended by construction of a transposon that carries AvrRpt2$^{81-255}$ and is capable of generating translational fusions with the N-terminal regions of candidates[58].

Finally, Cya (adenylate cyclase), which was originally used to demonstrate the ability of *Y. enterocolitica* to translocate YopE into mammalian cells[138], has been shown to be similarly useful in demonstrating the ability of the plant pathogen *X. campestris* pv. vesicatoria to translocate AvrBs2 into pepper leaf cells[31]. The Cya assay is based on the dependence of adenylate cyclase on the eukaryotic protein calmodulin for activity, thus permitting detection of the translocation of Cya-containing hybrids into plant cells. As expected, this system also works with *P. syringae* effectors, and we have used it to demonstrate the ability of *P. syringae* pv. tomato DC3000 to translocate several effectors into *Nicotiana benthamiana* cells (Schechter, Roberts, Jamir, Alfano, and Collmer, unpublished).

Despite the development of a medley of tools for studying the secretion and translocation of TTSS substrates in various pathogens, the nature of the signal remains controversial[95, 127]. This controversy is brought into sharpest focus with protein fusions involving the first 15 amino acids of *Yersinia* Yops with reporter proteins, such as Npt. The ability of these hybrids to be secreted by the TTSS despite frameshift mutations altering the Yop amino acids suggests that targeting information resides in the mRNA[6, 128]. However, wobble nucleotide mutations altering the mRNA argue against an mRNA signal, and the finding that synthetic amphipathic sequences of eight residues can function as a targeting signal suggests that targeting information resides in a relatively general property of the protein[96]. Perhaps these discrepancies can be reconciled on the basis of different contexts for the mutations and the possibility that both mRNA and amino acid signals are used[127], as suggested for the flagellar protein FlgM[84].

P. syringae effector targeting signals also have been explored in the context of the mRNA signal hypothesis. Notably, AvrPto$_{1-15}$-Npt is secreted by the *Y. enterocolitica* TTSS, and secretion is not disrupted by frameshift

mutations affecting the AvrPto sequences[5]. Although we expect the targeting process to work the same in the TTSSs of both plant and animal pathogens, we have been unable to directly test frameshifted $AvrPto_{1-15}$-Npt constructs in the plant pathogen Hrp TTSS systems of *E. chrysanthemi* or *P. syringae* because of the weaker secretion of the parent $AvrPto_{1-15}$-Npt construct and the far weaker secretion of all TTSS substrates by plant pathogens[5]. However, frameshift mutations affecting the first 18 codons in *avrBs2* did not abolish secretion by the TTSS of *X. campestris* pv. vesicatoria, suggesting the possibility of an mRNA targeting signal[107].

P. syringae researches have a special interest in TTSS targeting signals because the identification of such signals could enable prospecting for candidate effector genes in sequenced genomes. This is particularly important because the poor and variable secretion of effector proteins in culture precludes their identification in *P. syringae* culture supernatants, and apparent redundancy among effectors precludes their identification by screening mutants for loss of virulence. Two approaches have been taken to predicting TTSS targeting signals in novel *P. syringae* proteins. Both relied upon functional assays for generating a training set for pattern recognition. In one approach, the $AvrRpt2_{81-255}$ transposon was used to identify a set of 13 TTSS substrates in *P. syringae* pv. maculicola[58]. In the other approach, a Hidden Markov Model was used to identify a set of effector candidates in the *P. syringae* pv. tomato DC3000 draft genome based on the 5′ presence of HrpL-dependent promoters, and subsequent secretion/translocation tests then generated a training set of 28 known or newly confirmed TTSS substrates[49, 117]. Examination of these proteins revealed amino-acid biases in the first 50 residues, most notably high Ser content[58], and they also revealed a pattern of equivalent (based on solvent exposure) amino acids in the first 5 positions and a lack of acidic amino acids in the first 12 positions[117] (Figure 4). These patterns were then used to search the draft genome of *P. syringae* pv. tomato DC3000[58, 117]. Further rounds of experimental testing and genome analysis revealed additional effector genes in the DC3000 genome[35, 55]. As discussed in another chapter, the current inventory of TTSS substrates in the closed and annotated DC3000 genome includes 31 confirmed effectors and 19 candidates with multiple properties of effectors[27].

5.2. Chaperones for TTSS Substrates

Chaperones also play a role in the process of targeting some proteins to the TTSS. TTSS chaperones have been extensively studied in animal pathogens and only recently have been demonstrated in the plant pathogens *P. syringae* and *E. amylovora*[53, 156]. A striking feature of TTSS chaperones is that they may be dedicated to one or a few effectors[159]. Cognate chaperone and effector genes are typically linked, and chaperones may be predicted in

genomic sequences based on such linkage and on the general structural features of chaperones: small size, acidic isoelectric point, and predicted amphipathic α helix in their C-terminal regions[113]. Of the 50 confirmed or predicted effector genes in the DC3000 genome, 9 are linked with putative chaperones.

Chaperones variously stabilize effectors, prevent inappropriate interactions within the bacterial cell, and promote the translocation of effectors[113]. A current hypothesis, which awaits rigorous testing, is that chaperones establish a priority in the delivery of effectors, and specifically promote the rapid entry into the TTSS pathway of preformed pools of effectors[22, 113]. The *P. syringae* pv. syringae 61 HopPsyA effector is secreted in culture and elicits the HR *in planta* in a manner that is strongly dependent on the ShcA chaperone[156]. As is typical of animal pathogen chaperones, the *hopPsyA* and *shcA* genes are in the same operon, and affinity chromatography experiments involving truncated derivatives suggest that the chaperone-binding domain exists in the first 100 amino acids of HopPsyA[156]. More recently, the *P. syringae* pv. tomato DC3000 HopPtoM effector has been shown to be secreted and translocated in a manner dependent on its chaperone, ShcM[8].

6. DISSECTING STAGES IN THE JOURNEY OF TTSS EFFECTOR PROTEINS

We now have several snapshots of the journey of TTSS effectors from the cytoplasm of the pathogen to subcellular targets within host cells, and the sets of TTSS components controlling stages in the journey. For example, mutagenesis of the *hrcJ* and *hrpU* operons, which encode several Hrc proteins located in the inner membrane, prevents HrpZ from leaving the bacterial cytoplasm, whereas mutagenesis of *hrcC*, encoding the outer membrane secretin, results in accumulation of HrpZ in the periplasm[34]. Thus, protein movement across the bacterial envelope can be experimentally separated into two stages. Movement through the Hrp pilus represents a third stage that is required for both translocation of effectors *in planta* (as indicated by effector-dependent elicitation of the HR) and secretion of effectors and harpins in culture[130, 160]. In many pathogens, TTSS gene expression and secretion/translocation activity are interconnected[36]. For example, contact with host cells is often important in activating the TTSS, as observed with the plant pathogen *R. solanacearum*[101]. There is presently no evidence for contact-dependent secretion by the *P. syringae* TTSS. However, it is noteworthy that HrpA is rapidly induced *in planta*[21], and it is also required for the full expression of other *hrp* genes[160].

We have clues to other dynamic aspects of the TTSS process. For example, bacterial protein synthesis is required for 0.5–1.0 hr *in planta* for effector

delivery to occur, even if the Hrp system has been induced before inoculation (as indicated by eventual elicitation of the HR following treatment with different antibiotics)[125]. Compromising plant metabolism by treatment with heat or cycloheximide can differentially alter the subsequent ability of various TTSS mutants to elicit the HR[23]. Furthermore, different TTSS activities can be preferentially affected by environmental conditions. For example, cool temperatures favor *P. syringae* pathogenesis[72], and similarly, the secretion in culture of effectors (but not of the HrpZ harpin) is strongly dependent on incubation at 20°C rather than 30°C[155].

The final stage in the journey of effectors is to the appropriate subcellular location in plant cells. For example, AvrB, AvrRpm1, AvrPphB, and AvrP to carry N-terminal consensus eukaryotic fatty acylation signals, and these are required for biological activity[111, 136] (Figure 4). Furthermore, expression of these effectors in plant cells reveals that they are fatty-acylated and localize to the plasmamembrane. In other plant pathogenic bacteria, such as *X. campestris* pv. vesicatoria, members of the AvrBs3 family of effectors have been shown to localize to the host nucleus[145, 154]. There is presently no evidence that any *P. syringae* effectors are localized to the nucleus, however, many recently identified effectors await testing for localization to subcellular targets in plant cells.

7. A BROADER LOOK AT THE ROLE OF THE TTSS IN *PSEUDOMONAS* BIOLOGY

There are many unanswered questions about the ecological significance of the TTSS in *Pseudomonas*. If we consider that carriage and expression of a TTSS and associated proteins can potentially confer a substantial burden on bacteria in the absence of a susceptible host, three predictions are afforded regarding the distribution of TTSSs in natural populations. (a) We can expect pathogenic *Pseudomonas* to have an epidemiological population structure, in which bacteria diversify in the absence of a susceptible host, potentially accumulating loss of function mutations in TTSS and effector genes. However, the presence of a susceptible host, or conversion from avirulence to virulence through acquisition of appropriate combinations of effector genes will promote a clonal population explosion of virulent bacteria, followed by further diversification and selection for novel effectors and improved TTSS function. Agricultural practices and plant monoculture have favored the ecological success, global distribution, and high visibility of these "clonal" strains, but we still know very little about the ecology of *Pseudomonas* on wild plants. (b) The predicted mode of action of *Pseudomonas* TTSSs, in which TTSS-expressing bacteria change the physiology of host cells, and hence of their surrounding environment may promote evolution and invasion

by "cheaters," bacteria which fail to express a TTSS but take advantage of changes wrought in host cells by those that do, thereby getting the benefits of defense suppression and nutrient release without the cost. Coinoculation experiments with Hrp^+ and Hrp^- bacteria clearly indicate that this can happen[70]. Detailed studies of the genetic structure of bacterial populations within lesions, and of the microenvironments experienced by "active" and "passive" bacteria within lesions are needed to address what actually happens during infection. Studies of mammalian pathogens have shown that many pathogens accumulate mutations in key "early" virulence loci during later stages of infection when these loci are no longer necessary, or are actually detrimental for competitive fitness within the host[61]. (c) Also, it is clear that a limited number of effector proteins are widely distributed in many different bacteria. We must consider the possibility that some of these proteins have broadly conserved functions that can confer useful characteristics, or a limited degree of parasitism, even in a "nonhost" context. The presence of TTSSs in symbiotic and non-pathogenic bacteria clearly suggests that we need expand our perception of the TTSS as a pathogenicity mechanism to one of a host-interaction mechanism[37, 38, 46], and that we need to take a much closer look at what *P. syringae* and *P. fluorescens* are doing with their TTSSs when they are not interacting with plants.

In this regard, it is useful to consider *P. aeruginosa*, a multi-kingdom opportunist pathogen whose menagerie of potential hosts includes humans, insects, nematodes, amoebae, and plants[30, 124]. Although *P. aeruginosa* carries a TTSS, which is important in pathogenesis in mammals, insects, and amoebae[105, 124], we have not addressed *P. aeruginosa* in this chapter because there is little evidence of ecological adaptation for plant interactions or for a role of the TTSS in plant pathogenesis. For example, the *P. aeruginosa* TTSS is quite divergent from that of plant pathogens and lacks either harpins, HrpA, or AvrE-family homologs, which are widely associated with the Hrp TTSSs of plant-associated bacteria. Perhaps the TTSSs of plant bacteria are more specialized because of the need to penetrate the plant cell wall. This is a significant question that remains to be tested.

The importance of the Hrp TTSS in the plant pathogenicity of *P. syringae* is now established, but many fundamental questions about the operation of the system remain unanswered. How does the extracellular TTSS machinery breach the plant cell wall? How does the bacterial cell recognize substrates for the TTSS pathway? Is traffic through the pathway controlled with regard to the order in which proteins enter the pathway and the site at which they exit the pathway, for example, in the plant cell wall or inside plant cells? The answers to these questions should yield new insights into both the capabilities of bacterial TTSSs and the nature of *P. syringae* interactions with plants.

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REGULATION

7

GLOBAL REGULATION IN *PSEUDOMONAS SYRINGAE*

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1. INTRODUCTION

Two questions come rather quickly to mind when one is confronted with writing a chapter on global regulation in *Pseudomonas syringae*. The first of these is very basic, and goes to the eventual scope of the article: what is *P. syringae*? This is not a straightforward question, as the species is something of a taxonomic mess. *P. syringae* has long been recognized as a member of the original "inner circle" of pseudomonads, eventually codified as the rRNA Group I[47]. This group was further refined by both general and molecular characteristics into two additional phylogenetic groupings recently described as the intrageneric clusters (IGC) I and II, with *P. syringae* being in IGC II[63]. Detailed analysis of the nucleotide sequences of the *gyrB* and *rpoD* genes indicated that the IGC II cluster could be reduced still further to three complexes, with one being the "*P. syringae* complex" containing the pathovars and nomenspecies traditionally associated with *P. syringae*[63]. After this point, the taxonomic picture becomes less clear. Plant pathogenic pseudomonads are not readily differentiated by standard phenotypic characteristics and have often been classified largely by host range as pathovars or subspecies of *P. syringae*[53]. This is an unusually

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heterologous grouping, with DNA hybridization studies, ribotyping, and general characteristics having defined at least six[47], and possibly as many as nine[17], discrete genomospecies within the complex. Some of these have already been proposed to be elevated to species status[17], with more sure to follow. However, as there is no clear agreement as to what should go where, or when it should happen, this situation is not likely to be completely resolved any time soon. It is thus something of an open question as to what data should be included in this review. To simplify matters for this chapter, we have essentially defined *P. syringae* in the less strict sense of the complex described above, allowing the inclusion of information from a larger group of plant pathogenic pseudomonads.

The second question posed is more obvious, and once again goes to the scope of the article: what is global regulation? As with many conceptual terms in molecular biology, a strict definition for global regulation is made difficult by the constant influx of new data that stretches our previous understanding of the ability of bacterial cells to organize and delegate resources. The basic concept of global regulation can be fairly intuitive, one that relatively distinguishes between "local" regulators that effect the expression of a specific gene group often physically linked to the regulatory gene(s), and more broadly based regulators that coordinate the expression of multiple gene groups in response to cellular conditions. Thus *lacI*, the negative regulatory element of the lactose utilization operon, is not a global regulator, since the effects of its regulatory activity are largely confined to the *lac* operon. The gene *rpoH*, on the other hand, encodes a sigma factor required for the efficient transcription of a variety of unlinked genes that a bacterial cell needs expressed at elevated temperatures, and thus has global effects within the cellular milieu. To some degree, responsiveness to the environmental condition is usually implicit in discussions of global regulation. We rarely hear the *rpoD* (sigma70) gene product being described as a global regulator, although it clearly is one in that it controls the expression of a disparate lot of unlinked genes; this distinction appears to reflect the understanding that RpoD, as the major sigma factor, is simply part of the regular cell machinery under "normal" growth conditions. It is the cell's ability to coordinate specific gene expression in response to the peculiarities of its current situation that defines the global regulatory webs. In this review, we will describe data related to global regulatory elements that have been described in *P. syringae*. It is worth noting that in some cases global effects for a particular element have not been directly demonstrated in *P. syringae*, but were extrapolated from data in other systems such as *P. aeruginosa*. Also, while we have endeavored to make this chapter readable by itself, mechanistic details for some of the gene systems that will be covered extensively elsewhere in these volumes have been minimized to avoid overlap.

2. REGULATORS

2.1. Acyl-Homoserine Lactone

Quorum sensing is the ability of cells to detect relative population densities and respond appropriately. Most research in this area has revolved around a class of small molecules called acyl-homoserine lactones (AHSLs). Production of AHSLs usually involves two conserved proteins of the LuxR/LuxI regulatory family, with LuxR being a transcriptional regulator that is a ligand for AHSLs produced by the LuxI synthetase[16]. Once activated by binding AHSL, the LuxR protein can participate in the positive global regulation of a variety of cellular functions in response to the hormone gradient, including further expression of *luxI*. AHSLs are lipid soluble and can traverse the membrane by simple diffusion[16]. Many phytopathogenic pseudomonads have been shown to produce AHSLs. A screen of soil and plant isolates of different *Pseudomonas* species indicated that AHSL production is more common among plant-associated bacteria than among those from soil[13]. Forty percent of the *P. syringae* strains in the study tested positive for AHSL, which were largely short-chain varieties like *N*-hexanoyl-L-homoserine lactone, *N*-(3-oxo-hexanoyl)-homoserine lactone, and *N*-(3-oxo-octanoyl)-L-homoserine lactone. No consistent association of AHSL production with particular genomospecies was observed. Genes involved in quorum sensing have been cloned from *P. syringae* pv. syringae[12], *P. syringae* pv. tabaci , and *P. syringae* pv. maculicola[13]. All were similar to each other, and fit the general model described above. Mutants were constructed in *P. syringae* pv. syringae by marker exchange using a disrupted *ahlI* AHSL synthetase gene[12, 34]. The resulting strains were completely negative for detectable AHSL production by bioassay. In contrast to results obtained in other *Pseudomonas* species, no other phenotypes were observed in these mutants, which appeared to be qualitatively the same as wild type for antibiotic production, swarming activity, extracellular protease, alginate, hypersensitive response (HR) on tobacco, and virulence on bean (Kinscherf, unpublished data, and refs [12, 34]). However, recent preliminary evidence suggests that AHSL production does indeed have quantitative effects on a number of phenotypes in *P. syringae* pv. syringae, including a negative regulatory effect on conditional swarming (S. Lindow, personal communication).

2.2. AlgT/DsbA

Alginate production has been shown to provide bacterial cells with some measure of protection against a variety of environmental conditions, including heat, desiccation, and osmotic and oxidative stress[31]. This exopolysaccharide is

most well known for being associated with mucoid forms of *P. aeruginosa* isolated from human patients afflicted with cystic fibrosis[11]. In fact, however, alginate production is ubiquitous among the pseudomonads[49], and has been studied in some detail in several backgrounds including *P. syringae*[14]. Regulation of alginate production is complex, involving a variety of specialized regulatory genes[18], as well as several global regulators including the *gacS*/*gacA* two-component system[59]. One alginate regulatory gene encodes a sigma factor that is variously known as AlgT, AlgU, sigma-E, and σ^{22} (see ref. [24]). Recent results from *P. aeruginosa* demonstrated that, besides its effects on alginate gene expression, AlgT also regulated expression of the disulfide bond isomerase DsbA[54]. Regulating *dsbA* gene expression also effectively regulates a number of other processes since DsbA protein is involved in the proper folding of proteins transported to and through the reductive environment of the periplasm. Accordingly, insertion mutations in the *P. syringae dsbA* gene were found to affect a variety of wild-type phenotypes, including mucoidy, fluorescence, and virulence[38]. It would seem likely that regulation of DsbA expression by itself has the potential to extend AlgT's regulatory reach to a global scale.

The *algT* gene of *P. syringae* pv. syringae has been cloned and sequenced, with the deduced amino acid sequence of the gene product showing 90% identity with AlgT from *P. aeruginosa*[31]. Not surprisingly, mutations in *algT* rendered *P. syringae* defective for alginate production, and *trans* complementation of these mutations by wild-type *algT* genes from either *P. syringae* or *P. aeruginosa* was observed[31]. Transcription of *algT* was activated by heat shock and osmotic stress in both *P. aeruginosa* and *P. syringae*[31]. A difference between the two species was observed in the response of *algT* mutants to the oxidative agents paraquat and H_2O_2, with *P. syringae* mutants being sensitive to both, and *P. aeruginosa* mutants being sensitive to paraquat alone[31]. In addition, it was noted that copper, frequently used as an antimicrobial agent in agriculture, stimulates activity of the *algT* promoter in *P. syringae*[31]. This promoter activation was speculated to be part of the oxidative response to the generation of free radicals by copper ion. However, given the regulation of DsbA by *algT*, it seems possible that additional selective pressure for promoter activation might stem from heavy metal toxicity, since improperly folded proteins might be more sensitive to thiol-binding divalent metal cations. Although the *algT* operon is usually described as being highly conserved with a gene order of *algT–mucA–mucB–mucC–mucD*, in *P. syringae* the positive regulatory gene *mucC* has been found to be completely absent from the locus, while the relative position of the other genes is retained[32].

2.3. GacS/A

A screen of Tn*5* insertions in *P. syringae* pv. syringae B728a for mutants affected in the ability to form water-soaked lesions on bean pods resulted in

the isolation of a strain that was completely nonpathogenic on plants[61]. Since the mutation rendered the bacteria unable to form lesions, it was given the designation *lemA* (*le*sion-*m*inus). Subsequent experiments revealed that, unlike mutants in the *hrp* secretory pathway, *lemA* mutants retained the ability to grow on plants in growth chamber experiments[61]. Southern hybridizations indicated that the gene was widespread among isolates of *P. syringae* pv. syringae, and that its effects on virulence were conserved in this pathovar[50]. At the same time, the virulence role of *lemA* was shown to be pathovar specific, since *lemA* mutants of *P. syringae* pv. phaseolicola retained full pathogenicity and antibiotic production[50]. The *lemA* mutations in *P. syringae* pv. syringae were pleiotropic, affecting the production of the antibiotic syringomycin and extracellular protease[28], suggesting either regulatory or transport defects. This question was resolved by the DNA sequence analysis of the gene, which revealed that the predicted LemA polypeptide was the histidine kinase sensor portion of what was presumed to be a two-component regulatory system, since no obvious response regulator was linked to the *lemA* locus[27]. However, mutant screens had detected an apparent spontaneous mutant that shared the *lemA* phenotypes, with the additional characteristic of being sensitive to UV radiation[51]. This combination of pleiotropy with UV-sensitivity had also been described for the then recently discovered *gacA* gene in *Pseudomonas fluorescens*, with UV-sensitivity resulting from polar effects on a known SOS component, *uvrC*[39]. The *P. fluorescens gacA* had been shown to be a response regulator without a linked sensor gene, making it an apparent candidate for being the missing second component matched to *lemA*. Mating experiments demonstrated that the spontaneous mutant was restored by a plasmid carrying the *P. fluorescens gacA* gene[51]. The wild-type version of the *P. syringae* gene was isolated by homology to the *P. fluorescens* gene and a disrupted copy was used to replace the *gacA* gene in the chromosome of B728a. The phenotypic pattern of the resulting mutant was identical to *lemA* mutants with the exception of the UV-sensitivity associated with the *uvrC* gene that was also located downstream in *P. syringae*. Also, Southwestern (DNA–protein) hybridizations revealed a DNA binding protein that was similarly down-regulated in both *lemA* and *gacA* mutant backgrounds[51]. These genetic data were interpreted as strong evidence that *gacA* was the cognate response regulator for *lemA*.

Suppressor studies in B728a suggested that there might be at least two pathways subordinate to the *lem/gac* two-component hierarchy. Data showed protease production could be restored to *lemA* mutants by extra gene copies of the ribosomal proteins L35 and L20, the presence of which had no apparent effect on antibiotic production or virulence[37]. Similarly, the syringomycin deficiency of a *lemA* mutant was suppressed by increased copy numbers of the *salA* gene, encoding a small positive autoregulatory protein without significant similarity to known regulators[36]; the *salA* gene was not a suppressor

of the protease phenotype, and *salA* mutants were not deficient for protease production. Reporter studies confirmed that *lemA* and *gacA* were required for high-level *salA* expression, and that *salA* regulated the expression of the syringomycin biosynthetic gene *syrB*. Strikingly, *salA* mutants of B728a were as nonpathogenic on bean as *lemA* mutants[36]. Since insertional inactivation of genes involved in syringomycin biosynthesis had no apparent effect on pathogenicity in B728a, it appeared that there was a separation in the antibiotic and virulence phenotypes observed for *salA*[36]. More recent results indicated that *salA* was also involved in the regulation of an antibacterial activity in B728a thought to reflect production of the antibiotic syringopeptin[4]. Disruption of the *sypA* gene involved in syringopeptin biosynthesis resulted in elimination of this activity in bioassays and in a significant reduction in virulence on bean in growth chamber experiments. However, neither the *sypA* mutant, nor a *sypA–syrB* double mutant with no detectable antibiotic activity, were as affected in virulence as either *lem/gac* mutants or *salA* mutants, suggesting again that some other function(s) controlled by the *gac*/*sal* regulatory pathway was involved in the full virulence of B728a on bean (Kinscherf, unpublished data).

During this time period, the *lemA*/*gacA* two-component system was found in a wide variety of other species and genera, eventually establishing the gene pair throughout the gamma Proteobacteria[21]. In many of these cases, the basis for detection was the effect that mutation of one or both of the two components had on the production of extracellular factors involved in such fundamental processes as biocontrol, virulence, and biofilm formation. Nomenclature became more confusing, as the propensity for renaming what was clearly the *lemA/gacA* gene pair with new designations based on more taxonomically "local" phenotypes became widespread. In order to facilitate better continuity in the literature, as well as to make completely obvious the functional link between the two genes, we redesignated *lemA* as *gacS* and proposed that the designations *gacS* and *gacA* be generally implemented in reference to the gene pair[36]. The redesignation also had the advantage of allowing the system to be addressed under the collective heading of *gac*, or Gac. This nomenclature has been widely accepted by researchers, at least in genus *Pseudomonas*.

The list of phenotypes affected in *gac* mutants of phytopathogenic pseudomonads continues to expand and has retained the trend of affecting the expression of extracellular factors. Alginate[59], AHSL[34], tabtoxin[2], and syringolin production[58], as well as a swarming behavior[34], were all shown to be regulated by the *gac* genes in pathovars of *P. syringae*. Pectate lyase production required intact *gac* genes in *Pseudomonas viridiflava*[40, 41]. Coinoculation of *hrp* and *gac* mutants of *P. syringae* pv. syringae, all of which were independently nonpathogenic, promoted growth and lesion formation

by the *hrp* mutants in field studies[26], suggesting some type of functional extracellular complementation. The wide range of phenotypes affected in mutant strains are an indication of why virulence is also often affected in *gac* mutants, since parasitism of the host is an offshoot of the bacterium's ability to modify the local environment to its advantage. The degree to which any particular extracellular product contributes to this advantage is also a factor in the greater combined effect seen in *gac* regulatory mutants. As a corollary to this, since it is the specific host that determines the local environment for the parasite, the expression, or lack thereof, of virulence factors regulated by *gacS* and *gacA* may have varying effects depending upon the respective milieu.

A number of questions are unanswered concerning the mechanisms underlying *gac* regulation in bacteria. Determining a specific environmental signal that triggers the phosphorylization cascade initiated by the *gacS* sensor kinase remains to be done, as well as ascertaining whether the signal is the same in all genetic backgrounds. Also, while it seems clear that at least some of the regulatory influences manifested by *gacS* and *gacA* derive from their moderation of the *rsmABC* system affecting the stability of specific messenger RNAs[10], it is unclear whether the interaction with these post-trancriptional regulators is the sole access point for *gac* input within the cell.

2.4. GidA

The *gidA* gene was originally discovered simply as the ORF adjacent to the origin of replication *oriC* in *Escherichia coli*, and was so designated due to the filamentation phenotype observed for gene insertion mutants grown in glucose (*g*lucose *i*nhibited *d*ivision)[43]. Studies in a minichromosome system appeared to indicate that transcription starting at the *gidA* promoter affected the initiation of chromosome synthesis. This data, coupled with other information, led to a long-term understanding of *gidA* as a cell-division gene to the extent that the *gidA* locus was used as a marker to screen for origin of replications presumed to be linked to it[46]. More recent data suggested that the effects of *gidA* mutation on replication might be artifacts of the extrachromosomal locale used in minichromosome experiments[3]. However, in the absence of data indicating another function for the gene, the association of *gidA* with replication lingered. The predicted GidA gene product is essentially universal and highly conserved in the domains *Bacteria* and *Eucarya*, but is completely absent from members of domain *Archaea*[35].

Sequence analysis of chromosomal transposon (Tn*5*) insertions in *P. syringae* pv. syringae strain B728a selected for defects in virulence demonstrated that two insertions were in the *gidA* gene[52]. These mutations were pleiotropic, affecting not only virulence but also the production of the antibiotic syringomycin. Further experiments increased the list of phenotypes

associated with the *gidA* mutations to include production of a second antibiotic (presumed to be syringopeptin), production of fluorescent pigment, and the ability to swarm on semisolid agar media[35]. Microscopic studies determined that while filamentation was not apparent with *P. syringae* cells grown in glucose, aberrant cell sizes and shapes, as well as increased cell chain formation, were obvious regardless of carbon source. Recombinational replacement of a wild-type *gidA* gene with one disrupted with an antibiotic cassette gave rise to an identical phenotypic pattern as the transposon insertions. In all cases, wild-type phenotypes were restored after complementation with an intact *gidA* ORF on a plasmid. Reporter studies confirmed a role in gene expression, as *salA–lacZ* and *syrB–lacZ* fusions have dramatically reduced β-galactosidase activity in *gidA* mutants; once again, reporter activity was restored by an intact *gidA* ORF[35].

At about the same time that work on *gidA* in *P. syringae* was progressing in our own lab, researchers working with other organisms were also finding *gidA* in seemingly strange contexts. Studies in *E. coli* suggested that there are functional similarities between the gene products of *gidA* and *miaA*, a gene involved in modifying tRNA to enhance the stability of codon–anticodon interactions[45]. Other work in *E. coli* revealed that inactivation of either *gidA* or *mnmE* increased the frequency of translational frame shifts at particular sites in a transcript[6]. The *mnmE* gene product is involved in the hypomodification of some tRNAs. Finally, mutations in the *MTO1* gene in *Saccharomyces cerevisiae* were pleiotropic for the expression of several mitochondrial genes[8]. This yeast chromosomal gene encodes a mitochondrial protein that is orthologous to the *gidA* gene product. It was suggested that the yeast phenotypes were the result of less than optimal translational proofreading. All of these results coupled with our own data suggested that the apparent *gidA* global regulatory effects observed in *P. syringae* were the results of some unknown translational defect.

2.5. HrpL, HrpRS, HrpV

The HR is a reaction in plant tissue to challenge with an incompatible pathogen. The response is mediated by a set of genes generically described as *hrp* genes that are widely distributed in gram-negative plant pathogens. These genes are also absolutely required for growth and virulence in pathogenic interactions on compatible host plants, thus yielding the gene designation, *h*ypersensitive *r*esponse and *p*athogenicity. The *hrp* genes in *P. syringae* are found as a cluster spanning some 25 kb and encode components of a type III secretion system and related gene products such as avirulence and effector proteins[9]. Also in the cluster are regulators involved in controlling the expression of structural *hrp* genes, and as such, are examples of "local" regulators as described in

the introduction[9]. There is growing evidence, however, that the influence of at least some of the *hrp* regulators extends beyond the endpoints of the *hrp* gene cluster; this is an attractive concept from the standpoint of coordinating the myriad systems involved in the bacterial/plant interaction.

The *hrp* positive regulatory cascade in *P. syringae* typically consists of three components, the alternative sigma HrpL[62], and HrpR and HrpS[20], which both have similarity to RpoN-dependent promoter enhancer-binding proteins. The HrpL sigma factor initiates transcription at promoters containing a conserved sequence motif, and is essential for the expression of many, if not most, of the genes within the *hrp* gene cluster[62]. The promoter of the *hrpL* gene has been shown to be activated by HrpR and HrpS, which are coexpressed as a single transcript and then apparently form a heteromeric complex after translation[29]. Expression of HrpR and HrpS could be observed without concurrent *hrpL* promoter activation, implying the intervention of a negative regulatory influence[7]. A screen of transposon insertion mutants for strains with constitutive *hrpL* promoter activity yielded clones with inserts in the *P. syringae* gene for the Lon protease. Further experiments indicated a 10-fold increase in the half-life of the HrpR protein in the *lon* mutant background, suggesting that Lon degraded HrpR[7]. The breadth of the HrpL regulon was outlined by two separate studies that used a variety of techniques to examine global gene expression in *P. syringae* pv. tomato for some dependence on *hrpL*. The first study used promoter modeling analysis, minitransposon screening, and gene arrays, and resulted in the detection of numerous genes previously unknown to utilize *hrpL* regulation[15]. Most of these genes were identifiable from matches to the database, and many appeared to be virulence-related. The second study used promoter analysis, Northern blotting, and gene arrays to detect at least eight novel genes dependent on the *hrpS* gene; seven of the eight were also dependent on *hrpL*[64]. In both cases, many of the dependent genes appeared to be unlinked to the *hrp* gene cluster, reinforcing the essential notion of "globality". A further study used reporter promoter fusions to locate *P. syringae* pv. tomato genes that were induced upon infection of *Arabidopsis thaliana*[5]. Over 500 such genes were isolated, many of which were dependent on *hrpL* for expression. Seventy-nine fusions were sequenced, and, once again, many of the genes were identified as having known or potential roles in virulence. In addition to the regulators described above, the *hrp* region also encodes another negative regulator called HrpV[48, 55]. This regulator has been implicated in regulating the production of the toxin coronatine in *P. tomato* pv. tomato, but no specific mechanism was demonstrated.

2.6. Indole-3-Acetic Acid

Indole-3-acetic acid (IAA) is a signaling compound long studied in plants, which is also produced by many plant-associated bacteria and fungi[19].

The gene pair required for IAA production in *P. syringae* pv. syringae, *iaaM* and *iaaH*, was identified in a library of strain Y30 by homology to similar genes from *Pseudomonas savastanoi* (formerly *P. syringae* pv. savastanoi)[42]. Marker exchange of a transposon-disrupted *iaaM* gene, encoding the tryptophan-2-monooxygenase, created a mutant unable to produce the auxin. The mutant appeared to be affected for *in planta* growth, but was also deficient for syringomycin production relative to both the parental strain and to the mutant restored with a wild-type *iaaM* gene on a plasmid[42]. This suggests that IAA can globally regulate the expression of other factors produced by the cell.

2.7. RecA/RulAB/SOS

The *recA* gene has been studied in a wide variety of bacterial species as a primary agent of homologous recombination, as well as the starting point of a global gene expression cascade known as the "SOS response". Although generally thought of as a reaction to DNA damage by chemical agents or radiation, SOS induction actually occurs under any conditions in which DNA synthesis is inhibited, such as starvation, pH change, and moving into stationary growth[30]. The response begins with the activation of the RecA protein by binding single-stranded DNA found downstream of the DNA lesion. The activated protein then interacts with the negative regulatory element LexA, resulting in that protein's autocatalytic hydrolysis. The loss of LexA repressor results in the large-scale derepression of a number of genes, including *recA* itself, essentially amplifying the response. While the basic goal of SOS is cell maintenance, at least some portions of the DNA repair pathway have been shown to be "error-prone", resulting in the accumulation of genetic mutations. The ability of cells to rapidly produce a changed genetic repertoire in response to environmental stress is thought to have long-term positive effects on species adaptability.

Little research has been done on the SOS response in *P. syringae*, a somewhat surprising fact given that epiphytic bacterial species on plant surfaces are exposed to fairly high levels of UV radiation from sunlight. Still, the *recA* gene was one of the earlier genes cloned from *P. syringae*[25], and eventually was mutated to produce recombination deficient strains[60]. Defects in *recA* were found to greatly decrease the level of UV resistance in mutants but have no effect on the bacteria's ability to grow on plants or cause disease symptoms in growth chamber experiments. More recent studies on UV resistance in *P. syringae* indicated that plasmid-borne genes can be involved in the process[56]. Sequence analysis revealed that the plasmid genes responsible for increased radiation resistance, designated *rulA* and *rulB*, are similar to the error-prone mutagenic pathway genes *umuD* and *umuC* in *E. coli*[56], and were subsequently shown to functionally complement an *E. coli* strain deleted

for the *umuDC* locus[33]. The *rulAB* genes are widely distributed on plasmids in various strains and pathovars of *P. syringae*[57]. Similar plasmid genes are also well known in other bacterial genera. The *rulAB* locus contains a consensus LexA binding site within its promoter, and the genes are effectively cryptic in a *recA* mutant background[56]. Also, the genes appear to be induced in response to UV irradiation, suggesting that *rulA* and *B* are indeed part of the bacterial SOS response[33]. Mutants in *rulB* were found to have their UV-mediated mutability to rifampicin resistance reduced by 12–14-fold, consistent with a disruption in error-prone repair. It is interesting to speculate that one evolutionary response to a high-flux UV environment might be to increase the number of error-prone repair genes. In this regard, it is worthwhile to note that a plasmidless strain of *P. syringae* pv. syringae, B728a, has at least two chromosomal copies each of *umu*-like genes (ORFs Psyr 0630, 2210, 3298, and 4058). The frequent occurrence of UV resistance elements on multicopy plasmids might reflect this tendency, with exposure to the near-UV in sunlight as a steady selective pressure. What makes this scenario particularly thought-provoking is the hypothesis that *rulAB* can serve as an integration hot-spot for genes involved in the pathogen–host interaction[1]. As such, plasmids containing the *rulAB* genes could have a powerful potential as vehicles for the horizontal transfer of genes affecting host specificity and virulence.

There is currently no published data linking the UV resistance associated with *rulAB* with that affected in *rpoS* mutants in *P. syringae*[44]. Also, the Lon protease, a well-established member of the SOS pathway, has been shown to be a negative regulator of the *hrp* pathway[7] and is discussed in Section 2.5.

2.8. RpoN

The sigma factor RpoN (also known as sigma-54) regulates the initiation of transcription for bacterial genes expressed in response to nitrogen availability. The *rpoN* gene was cloned from *P. syringae* pv. maculicola strain ES4326 and was used in a marker exchange protocol to construct an insertion mutant[23]. As expected for an *rpoN* mutant, the new strain was unable to utilize a number of nitrogen sources and could not grow at low levels of ammonia. In addition, production of the antibiotic coronatine was affected, with reporter studies demonstrating that expression of the biosynthetic genes for the intermediate coronamic acid was greatly reduced. The *rpoN* mutant could not grow or cause symptoms on *A. thaliana*, and did not elicit HR on tobacco. Constitutive expression of *hrpL*, a gene known to be involved in both coronatine production and HR (see above), partially alleviated some *rpoN* mutant phenotypes, including the induction of defense genes in a compatible host plant. However, the expression of *hrpL* was insufficient to restore *in planta* growth[23]. Further reporter studies in the *rpoN* mutant confirmed the down-regulation of

genes suspected of being causal to the observed phenotypes[22]. Constitutive production of *hrpL* restored at least some of the down-regulated genes to wild-type levels. The constitutive *hrpL* construct did not act as a suppressor of the coronatine-minus phenotype in *rpoN* mutants of *P. syringae* pv. maculicola, indicating that other components of the antibiotic pathway were affected[22]. Another independent study confirmed the reliance of the coronatine pathway on *rpoN*[55].

2.9. RpoS

The *rpoS* gene encodes the sigma-38 transcription initiation factor that regulates the expression of genes during times of starvation and stationary growth. While *rpoS* is often alluded to in reference to systems in *P. syringae*, a review of the literature indicates that there is surprisingly little primary data on the subject. One study examined the effects of *rpoS* mutation on resistance to the UV component of sunlight, and found that cell survivability decreased 1,000-fold in *P. syringae* pv. syringae B728a lacking an intact gene[44]. No basis for this increased sensitivity was demonstrated, although *rpoS* in other backgrounds has been shown to regulate repair genes. In our own lab, an *rpoS* mutation was introduced into the *P. syringae* pv. tomato strain DC3000, but no qualitative effects were observed for virulence on *Arabidopsis* (Willis, unpublished data).

3. CONCLUSION

While an impressive amount of data concerning global regulation has originated in *P. syringae* in the past couple of decades, it is difficult to tie this information together into a well-ordered cellular regulatory web. There are too many gaps in our knowledge base that cause the relationships between the individual threads of the regulatory pathways to remain unclear. Still, the information derived from *P. syringae* has made a significant contribution to the cross-disciplinary science of gene expression, just as researchers in *P. syringae* have benefited greatly from the data produced in other bacterial backgrounds. Well-established genetic systems like *P. syringae* pv. syringae B728a and *P. syringae* pv. tomato DC3000 are now fully sequenced, and this underpinning of genomics should greatly facilitate the design and accomplishment of future experiments. Further examination of global regulation in *P. syringae* is almost certain to yield some interesting surprises, given the uniqueness of the plant-associated environment that is home to the bacteria.

Note added in proof: While this volume was being edited, work was published (Chatterjee et al., *Mol. Plant. Microbe Interact.* 2003 Dec; 16(12):1106–17) that provided an overview of the Gac regulon in *P. syringae* pv. tomato, based on differentials between wild-type and *gacA* mutant backgrounds.

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8

POST-TRANSCRIPTIONAL REGULATION IN *PSEUDOMONAS* SPP. VIA THE Gac/Rsm REGULATORY NETWORK

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1. INTRODUCTION

The pseudomonads are ubiquitous bacteria found in diverse environments, and as a consequence they exhibit versatile nutritional and metabolic capabilities. Rapid detection of, and adaptation to, environmental conditions that can change swiftly are essential for efficient growth, colonisation and survival. At the genetic level such adaptive mechanisms can operate at the transcriptional, translational and post-translational levels and global gene regulatory networks may incorporate elements operating at any or all of these levels. Signal transduction pathways coupling the sensing of an environmental signal to modulation of the expression of the relevant target genes frequently involve the use of two-component systems, consisting of sensor histidine kinases coupled to their respective response regulators[19, 31, 35, 42]. The ever increasing number of bacterial genomic sequences becoming available has highlighted

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the diversity and distribution of such two-component systems. For example, analysis of the genome of *Pseudomonas aeruginosa* has revealed the presence of some 64 potential two-component systems, reflecting the capacity of *P. aeruginosa* to readily adapt to a wide variety of environmental niches[39]. Among these, the GacS/GacA two-component system, which is widespread in *Pseudomonas* spp. and other gram-negative bacteria (see ref. [15] for genetic symbols used for *gacS* and *gacA* in various other bacteria), is involved in controlling the production of antibiotics, hydrogen cyanide, exoenzymes, quorum sensing signal molecules and other traits related to virulence and ecological fitness (reviewed in ref. [15]). In plant-beneficial species of pseudomonads, *gac* mutants typically exhibit partial or complete loss of biocontrol capabilities, whereas in plant- or animal-pathogenic species of pseudomonads and enteric bacteria, *gac* mutants show reduced virulence[15]. For example, *P. aeruginosa gacA* mutants are attenuated in plant (*Arabidopsis*), burned-mice, nematode (*Caenorhabditis elegans*) and wax moth larvae infection models[21, 36, 45]. Typically, these processes depend to a great extent on the synthesis of exoproducts (secondary metabolites and/or enzymes). The designation "Gac," that is, *g*lobal *ac*tivator of secondary metabolism, originated from the observation that *Pseudomonas fluorescens gacA* mutants do not produce antibiotics and cyanide, and consequently have a reduced ability to protect plants against a variety of pathogenic fungi[25]. GacS is a membrane-associated, histidine sensor kinase considered as unorthodox because it carries a phosphoryl transmitter, a receiver and a histidine phosphotransfer (Hpt) output domain[15, 31, 53]. By analogy with other related sensor kinases[24, 31, 47], it is likely that upon activation by an unknown signal, GacS is first autophosphorylated and, by a phosphorelay mechanism, the phosphate is subsequently transferred to the response regulator GacA[15, 53]. This response regulator has a C-terminal helix–turn–helix DNA-binding domain and belongs to a family of proteins in which NarL of *Escherichia coli* and FixJ of *Sinorhizobium meliloti* are the best characterised examples[3, 5, 15, 42]. Phosphorylated GacA is assumed to have DNA-binding activity similar to that described for FixJ[10]. However, the target DNA sequence to which activated GacA binds is so far unknown.

Induction of Gac-dependent phenotypes is observed in bacterial batch cultures, and is particularly evident in the late exponential or early stationary phase of growth[6, 9, 22, 37]. Moreover, cell-free culture extracts from certain wild-type strains (e.g., *P. fluorescens* CHA0) have the ability to advance and enhance the level of expression of Gac-dependent genes and, although the chemical identity of the signal molecule(s) remains elusive, it does not appear to be related to well-established quorum sensing signal molecules such as the *N*-acylhomoserine lactones[14, 53] (AHLs). Thus, the GacS/GacA system may itself constitute part of a quorum sensing-like mechanism that controls exoproduct formation. In addition to the signal molecule(s) produced by the

bacterial cells, it has been reported recently that sugar beet exudates contain a compound that activates the synthesis of the lipopeptide amphisin only when the *gacS* gene of *Pseudomonas* sp. strain DSS73 is functional[23]. Thus, the GacS/GacA system may also serve to integrate and coordinate bacterial responses to environmental conditions by sensing chemically and structurally related signal molecules originating from a host organism.

A distinctive feature of the GacS/GacA two-component system resides in the mechanisms by which control of the expression of target genes occurs. This is essentially via a post-transcriptional regulatory network which incorporates RNA-binding proteins and small, untranslated regulatory RNAs. Genes belonging to this system have been designated *rsm* in *Erwinia* and *Pseudomonas* spp., for *r*egulators of *s*econdary *m*etabolism, and *csr* in *Escherichia, Salmonella* and other genera, for *c*arbon *s*torage *r*egulators.

2. GLOBAL POST-TRANSCRIPTIONAL CONTROL IN *P. FLUORESCENS* CHA0

In the plant-beneficial strain *P. fluorescens* CHA0, the GacS/GacA system positively controls the expression of genes required for biocontrol (e.g., *phlACBDE* for 2,4-diacetylphloroglucinol and *hcnABC* for hydrogen cyanide synthesis) and for extracellular enzymes (e.g., *aprA* for protease and *plc* for phospholipase C; reviewed in ref. [12], [13]). In the case of the *hcnABC* operon, which encodes hydrogen cyanide synthase (see Pessi and Haas, Vol. 3, Chapter 22), the regulation is post-transcriptional, that is, at the mRNA level, and involves the translational regulatory proteins RsmA and RsmE[6, 13, 14]. Evidence for this mechanism comes from measurements of the expression of transcriptional and translational reporter fusions controlled by the native or a constitutive promoter, in different genotypic backgrounds (Figure 1). This model is further supported by the observation that point mutations in the vicinity of the *hcnA* ribosome-binding site (RBS) can completely abolish Gac/Rsm control of *hcn* expression in *P. fluorescens*[6]. Similar mechanisms for controlling gene expression have been found for other GacS/GacA targets such as the *apr* and *phl* operons[13, 14] (C. Gigot-Bonnefoy and D. Haas, unpublished results). The current model proposes that GacS/GacA activates expression of target genes at the post-transcriptional level by inducing the transcription of at least two small, untranslated regulatory RNAs, termed RsmY and RsmZ, which alleviate the repressive effects of RsmA[14, 48] and probably also of RsmE (C. Valverde and D. Haas, unpublished results).

RsmA is a small, 6.9-kDa polypeptide considered to bind to the mRNAs of target genes blocking their translation[6]. The deduced amino acid sequence of RsmA contains a KH motif (consensus VIGKKGffIR, VLGVKGNQVR for

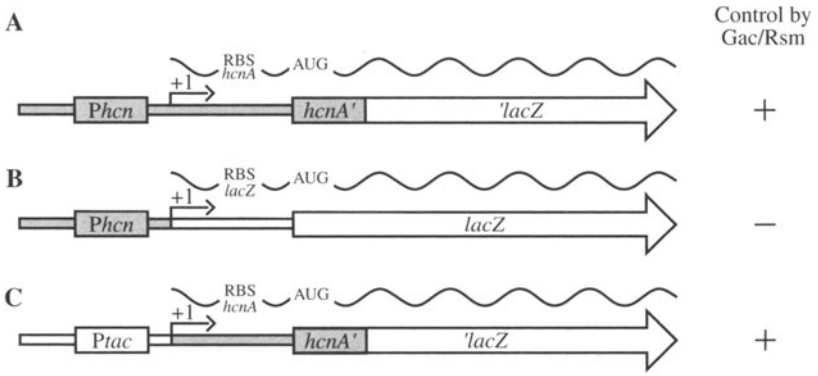

Figure 1. Gac/Rsm control of *hcnA*-based *lacZ* reporter fusions. (A) Expression of a translational *hcnA'–'lacZ* fusion under the control of the native *hcn* promoter is Gac-dependent and controlled by the Rsm system. (B) When *lacZ*, with its own ribosome-binding site (RBS) and untranslated leader region, is fused to the +1 site of transcription of the *hcnA* gene, promoter activity is reported; this fusion is Gac-independent and not affected by the Rsm system. (C) Replacement of the *hcn* promoter by the *tac* promoter in the *hcnA'–'lacZ* translational fusion produces a reporter construct that is controlled by Gac and Rsm, indicating post-transcriptional control at the mRNA level. Point mutations around the RBS indicate that regulation occurs in this region. This experiment has been repeated with the *hcnA* gene of *P. aeruginosa*, with similar results[33] (see also Pessi and Haas, Vol. 3, Chapter 22).

strain CHA0; see also Section 4) which is often found in RNA-binding proteins[11, 26]. Mutational analysis of the *hcnA* untranslated mRNA leader region suggests that about 10 nucleotides surrounding the Shine–Dalgarno sequence (GGA) are involved in the recognition by RsmA and in the regulation by GacA[6] (K. Starke and D. Haas, unpublished results). However, specific binding of RsmA to *hcnA* leader mRNA and the postulated inhibition of translation initiation remain to be demonstrated experimentally. A second regulatory protein, RsmE, ~7.0 kDa and sharing 71% identity with RsmA (Section 4), has been recently identified in strain CHA0[13] (C. Reimmann, unpublished results). Thus, these small proteins together may negatively control the translation of mRNAs such as those of the *hcn, apr* and *phl* operons during the exponential growth phase, via a common mechanism; when the GacS/GacA system is properly activated, regulatory RNAs intervene to relieve the translational repression of these target genes by forming stable ribonucleoprotein complexes (Figure 2).

In *P. fluorescens* CHA0, the riboregulator genes *rsmY* (118 bp) and *rsmZ* (127 bp) are transcribed in a cell density-dependent manner, and strictly require the GacS/GacA two-component system for their transcription[14, 48]. Whether the induction of transcription of these riboregulators by GacA is direct or indirect is not yet known. Addition of supernatant extracts from wild-type spent culture medium advances and enhances the expression of

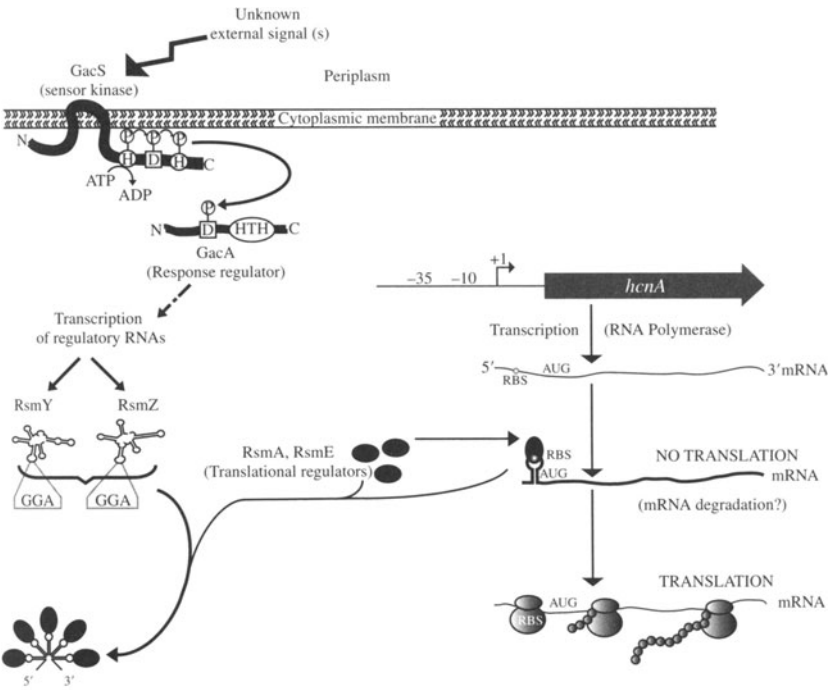

Figure 2. Proposed pathway of signal transduction involving the post-transcriptional control system RsmAE/RsmYZ in *P. fluorescens* strain CHA0. Activation of the sensor kinase GacS by unknown signals triggers a phosphorelay cascade between GacS and GacA which results in the activation of the response regulator GacA. Its helix–turn–helix motif is abbreviated HTH. Phosphorylated GacA positively controls the transcription of regulatory RNA(s) such as RsmY and RsmZ. These regulatory RNAs, whose most notable characteristics are conserved unpaired GGA motifs, are proposed to sequester translational repressors such as RsmA and RsmE, rendering the RBS of target mRNAs accessible for translation. Note that induction of *rsmY* or *rsmZ* transcription by GacA may be indirect and may require additional factors.

rsmY–lacZ or *rsmZ–lacZ* fusions, indicating that this system can sense extracellular signals. Interestingly, RsmA is also involved as a positive control element in the up-regulation of *rsmY* and *rsmZ*, via an unknown mechanism[48]. Overexpression of *rsmY* or *rsmZ* under the control of the *tac* promoter phenotypically suppresses *gacS*- and *gacA*-negative mutations[14, 48]. Conversely, overexpression of *rsmA* or *rsmE* in the wild-type strain mimics the phenotypes associated with *gacS* or *gacA* mutations[6, 14] (C. Reimmann, unpublished results). This indicates that RsmY and RsmZ are positive effectors that promote the translation of GacS/GacA-dependent genes by antagonising the effects of the translational repressors RsmA and RsmE. Binding of the RsmA

or RsmE proteins to RsmY or RsmZ RNAs have been observed in vitro[48] (our unpublished results). Thus, a model for global post-transcriptional regulation by the Gac/Rsm cascade in strain CHA0 can be proposed (Figure 2). Additional support for this model comes from similar systems in enteric bacteria. In *E. coli*, the CsrA (RsmA) protein can be sequestered by the two regulatory RNAs, CsrB and CsrC, thereby relieving the negative effect of CsrA on target genes[4, 40, 50]. Homologues of the GacS/GacA system positively control the expression of CsrB and CsrC in *E. coli*, and the expression of RsmB in *Erwinia carotovora*[7, 20, 44, 50]. Furthermore, in *Salmonella typhimurium*, the effect of a *barA* (*gacS*) mutation can be suppressed by overexpressing an *E. coli* CsrB homologue[2].

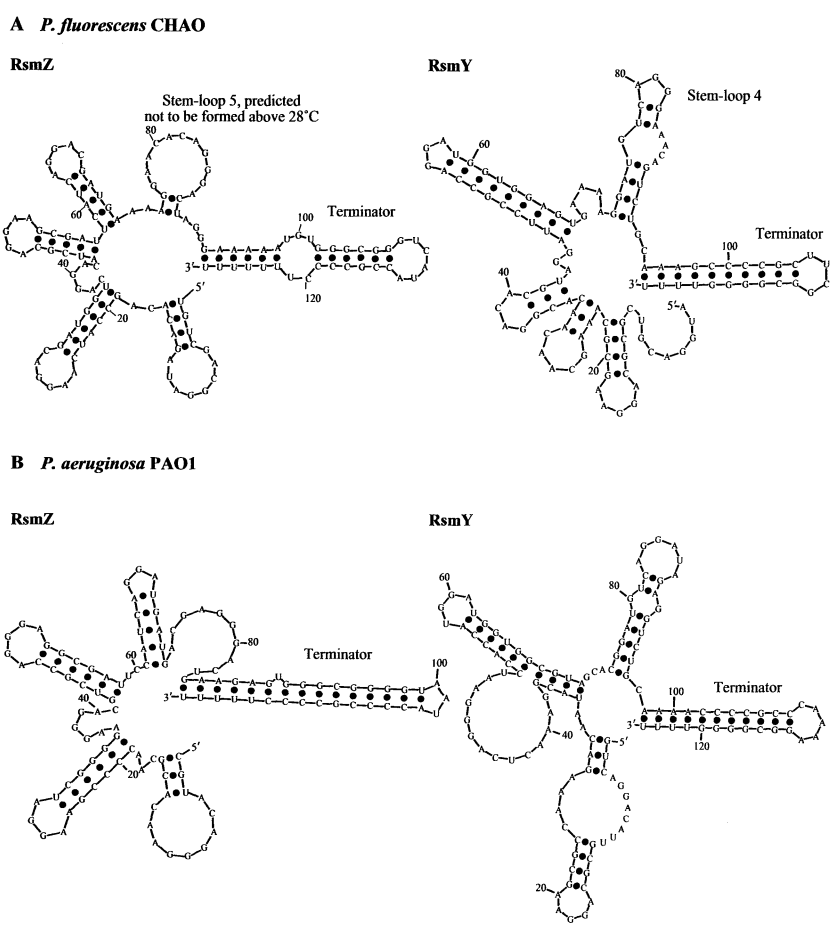

Figure 3. Predicted structures of RsmY and RsmZ (A) from *P. fluorescens* and (B) from *P. aeruginosa* using the mfold program[54]. The molecules present similar structures, forming stem–loops with conserved GGA motifs in single-stranded regions, and end with a rho-independent terminator.

RsmY and RsmZ (also known as PrrB in *P. fluorescens* F113)[1] exhibit some structural similarities and sequence features to riboregulators antagonising RsmA (CsrA) in other organisms such as CsrB and CsrC in *E. coli*, and RsmB in *E. carotovora*. For example, several GGA motifs can be found in the hairpin loops and in other single-stranded regions[1, 7, 14, 40, 48, 50] (Figure 3), although the overall sizes and the nucleotide sequences for these RNAs are quite different.

Deletion of either *rsmY* or *rsmZ* in the wild-type strain CHA0 has little effect on the production of extracellular metabolites, whereas an *rsmY rsmZ* double mutant has a strongly reduced production of 2,4-diacetylphloroglucinol, hydrogen cyanide and exoprotease. However, the *rsmY rsmZ* double mutant still produces these metabolites in significant amounts, compared to the basal levels in *gacS* or *gacA* mutants[48], suggesting that more than two GacA-controlled regulatory RNAs may exist in *P. fluorescens*.

The Gac/Rsm system can be locked in the "on" position by a deletion of the GacS linker domain, which is located between the second transmembrane segment and the autophosphorylation site of GacS. This deletion removing 76 amino acids residues from GacS causes elevated levels of *hcnA, aprA* and *phlA* expression and insensitivity of strain CHA0 to added signal[53]. The simplest, but by no means the only interpretation of this finding is that the linker domain of GacS may be important for both signal perception and signal transduction[53].

3. GLOBAL POST-TRANSCRIPTIONAL CONTROL IN *P. AERUGINOSA* PAO1

The regulatory circuitry for the production of exoproducts in *P. aeruginosa* PAO1 is more complex than in *P. fluorescens* CHA0, as the quorum sensing system relying on the two AHLs, that is, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL)[52], and the *Pseudomonas* quinolone signal (PQS)[8, 32] add an additional layer of regulation. In addition, the quorum sensing system depends on Gac/Rsm global post-transcriptional control[33, 34, 37]. However, whereas *P. fluorescens* CHA0 has two post-transcriptional regulatory proteins (RsmA and RsmE) in the Gac cascade, *P. aeruginosa* PAO1 has only RsmA. This is a 6.9-kDa protein sharing 84% identity with the homologous protein from *P. fluorescens* CHA0 and 92% identity with CsrA from *E.coli*. RsmA from *P. aeruginosa* PAO1 can functionally complement an *E. coli csrA* mutant[34]. The gene encoding RsmZ, a riboregulator antagonising RsmA, was located, as in other *Pseudomonas* species, immediately downstream of *rpoS*[18] (see also Section 4). RsmZ from *P. fluorescens* CHA0 and *P. aeruginosa* PAO1 are 127 and 117 nucleotides long,

respectively (Figure 3), and share 60 % nucleotide sequence identity. RsmZ has predicted secondary structures that are very similar to those of its *P. fluorescens* homologue[18] (Figure 3). Similarly, a homologue of *P. fluorescens* CHA0 *rsmY* has been found in *P. aeruginosa* (B. Humair, C. Valverde and D. Haas, unpublished results) and other pseudomonads, by homology searches and analysis of conserved flanking regions[48] (see also Section 4). RsmY from strains CHA0 and PAO1 are 118 and 124 nucleotides long, respectively, and share 72 % identity.

A model for regulation by the Gac/Rsm system in *P. aeruginosa* is proposed in Figure 4. One of the upstream components of this signal transduction pathway is the alternative sigma factor RpoN (σ^{54}), which negatively controls the expression of *gacA*, probably via an unidentified repressor[17] (Figure 4). RpoN regulates the transcription of a wide variety of genes, often in concert with transcriptional activators belonging to two-component regulatory systems, facilitating specific responses to environmental signals[41]. In *P. aeruginosa*, 14 potential transcriptional regulators contain RpoN recognition sequences, and 8 out of the 64 predicted two-component systems are members of the NtrC activator family and assumed to interact with RpoN[39, 43]. RpoN is known to regulate the expression of many catabolic genes in response to various carbon and nitrogen sources[29, 38]. This suggests that, in addition to responding to GacS phosphorylation, GacA activity can also depend on GacA concentration, which in turn might

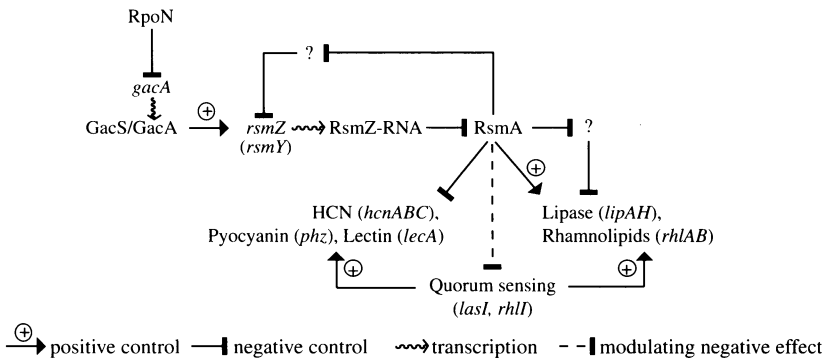

Figure 4. Proposed pathway of signal transduction involving the post-transcriptional control system RsmA/RsmZ in *P. aeruginosa* PAO1. GacA, itself under negative control by the alternative sigma factor RpoN, is necessary for the expression of *rsmZ*, and probably *rsmY*, coding for the riboregulators antagonising RsmA. RsmA positively controls *rsmZ* expression, probably by repressing an unknown repressor, thus forming a negative autoregulation circuit. RsmA also negatively controls quorum sensing and some genes encoding exoproducts; these are regulated indirectly at the transcriptional level via quorum sensing, but probably also directly at the translational level, as is the case for *hcnA*[33]. Lipase and rhamnolipid production are controlled positively by RsmA, directly or indirectly, via a negative control element.

be determined by the nutritional status of the cells with the help of RpoN. Furthermore, unknown signals from *P. aeruginosa* or from other organisms can stimulate GacS[15, 53]. GacA is essential for *rsmZ* and *rsmY* expression[18] (and our unpublished results), as found for *rsmZ* and *rsmY* in *P. fluorescens* CHA0[14, 48] and for the *rsmZ* homologue *prrB* from *P. fluorescens* F113[1].

The expression of *rsmZ* and *rsmY* in *P. aeruginosa* is cell population density-dependent, and occurs during late exponential phase of growth, as revealed using transcriptional *rsmY–lacZ* and *rsmZ–lacZ* fusions[18] (our unpublished results). *P. aeruginosa rsmZ* or *rsmY* mutants do not show significant phenotypic changes when compared with the wild type, except for a small increase in *rhlA* expression and rhamnolipid production in an *rsmZ* mutant[18] (our unpublished results). However, as observed for *P. fluorescens*, overexpression of *rsmZ* leads to major phenotypic changes similar to those observed in an *rsmA* mutant (see below), suggesting that at least RsmZ interacts with RsmA to modify the translational levels of target mRNAs. As in other organisms[48,50], RsmZ and RsmY from *P. aeruginosa* could inhibit RsmA activity by titrating out this regulatory RNA-binding protein.

In *P. aeruginosa* the expression of *rsmA* increases as a function of cell population density and this effect is independent of the GacS/GacA system[34]. An *rsmA* mutant forms smaller colonies than does the wild type. This phenotype could result from the mutant's inability to swarm[18], but perhaps also involves an altered metabolism, similar to the situation in *E. coli* where CsrA plays an important role in biofilm formation and the regulation of carbon storage[30]. Overexpression of *rsmA* was found to have extensive pleiotropic effects as it negatively controls the expression of the *lasI* and *rhlI* quorum sensing genes and, consequently, the production of 3-oxo-C12-HSL and C4-HSL, as well as the biosynthesis of many quorum sensing-dependent exoproducts (lectin, elastase, hydrogen cyanide, pyocyanin and staphylolytic enzyme). The effects of RsmA on the quorum sensing regulators *lasR* and *rhlR* appear to be minor[33, 34]. A detailed study of the mechanisms underlying the regulation of the *hcnA* gene revealed that the regulation of the *hcn* operon occurs at the transcriptional level via the LasR/3-oxo-C12-HSL and RhlR/C4-HSL quorum sensing circuitry. However, a second layer of control is exerted directly at the post-transcriptional level by RsmA[33, 34]. The *P. aeruginosa hcn* operon is therefore under the control of two parallel regulatory pathways by the Gac/Rsm system, only one of which involves AHL-dependent regulation. This dual mode of action may provide a way to control rapidly and differentially the production of hydrogen cyanide.

From an experimental point of view, these multiple layers of regulation complicate the interpretation of data obtained from mutants blocked in single elements of the pathways and it can be difficult to separate direct from indirect effects. For example, rhamnolipid production and swarming as well as lipase production are positively regulated by RsmA[18] (Figure 4). The positive effect

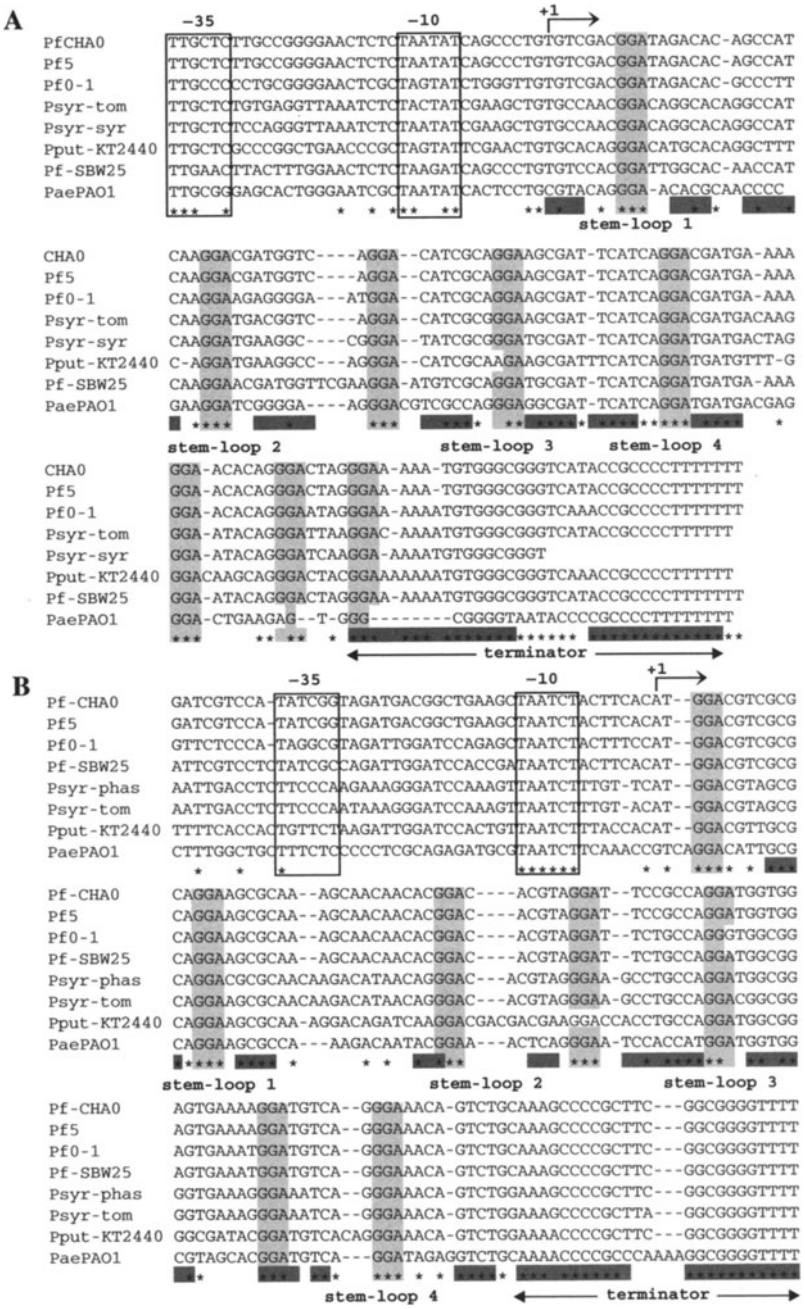

Figure 5. Continued

of RsmA could result either from enhanced transcript stability, as proposed for certain flagellar and other swimming motility-related genes in *E. coli* and *Erwinia*[28, 49], or from RsmA-mediated repression of an unidentified translational repressor (Figure 4). The overall positive effect of the Gac regulon on lipase expression is only partially accounted for in this model, in that GacA is a positive control element of lipase production, presumably by activating AHL-dependent expression of the type II secretion apparatus, which is required for lipase expression[37].

4. THE RSM SYSTEM IN OTHER PSEUDOMONADS

Whereas the GacS/GacA two-component system has mostly been described in pseudomonads and enteric bacteria (reviewed in [15]), CsrA and RsmA homologues appear to be widely distributed among the eubacteria[40, 51], suggesting that similar global post-transcriptional regulation pathways may be widespread, although they may also possibly be part of different regulatory cascades. Besides *P. fluorescens* and *P. aeruginosa, Pseudomonas syringae* also has a functional RsmA-mediated post-transcriptional control system (A. Chatterjee, Y. Cui., G. Nong, Y. Li, A. Collmer, and A.K. Chatterjee *Abstracts of the 11th International Congress on Molecular Plant–Microbe Interactions*, St. Petersburg, 2003). It is likely that all CsrA and RsmA homologues are orthologues derived from a common ancestor whose sequence and function has been relatively well conserved during evolution. In all the *Pseudomonas* genomes where *rsmA* has been found so far, it is located immediately downstream and transcribed in the same orientation as an ORF coding for a LysC aspartokinase, and upstream of a region coding for at least three tRNAs (usually *serV, argV*α and *argV*β). In enteric bacteria (e.g., *Escherichia coli, Yersinia pestis, Salmonella enterica* serovar *typhi, Erwinia chrysanthemi*), *csrA* (*rsmA*) is not found downstream of *lysC*, but it is also located upstream of three or more tRNAs, supporting the hypothesis of a common origin of the region.

In *Pseudomonas* spp., *rsmZ* homologues are found in the *rpoS–mutS* intergenic region, a locus suggested to have experienced frequent horizontal

Figure 5. Continued
Alignment of (A) *rsmZ* and (B) *rsmY* sequences from pseudomonads, produced by CLUSTAL W[46]. Potential transcription start points and −35 and −10 boxes are indicated. Experimental determination of the transcription start sites have been reported for the *rsmZ* homologue gene *prrB* in strain F113[1] and for *rsmY* in strain CHA0[48]. Rho-independent terminators are shown under the sequences. GGA motifs (shadowed) are characteristic of RsmA-antagonising riboregulators, and are found essentially in predicted single-stranded regions. Abbreviations: CHA0, *P. fluorescens* CHA0; Pf5, *P. fluorescens* Pf-5; Pf01, *P. fluorescens* Pf0-1; Psyrtom, *P. syringae* pv. *tomato*; Psyrsyr, *P. syringae* pv. *syringae*; PputKT2440, *P. putida* KT2440; PfSBW25, *P. fluorescens* SBW25; PaePAO1, *P. aeruginosa* PAO1.

gene transfers in the enterobacteria for the acquisition of virulence-related genes[16, 27]. It is therefore possible that a *Pseudomonas* species originally acquired *rsmZ* via horizontal gene transfer. Similarly, *rsmY* homologues can be found in several *Pseudomonas* genomes (Figure 5). With few exceptions (e.g., in *P. syringae* pv. tomato), the *rsmY* gene is located upstream and oriented in the opposite direction of an ORF coding for a putative transcriptional regulator similar to *P. aeruginosa* PA0528. Upstream of *rsmY* there is an ORF similar to PA0508, a putative acyl-CoA dehydrogenase (data not shown).

Whereas *P. aeruginosa* and enteric bacteria have a single *rsmA* (*csrA*) gene, some *Pseudomonas* spp. have two *rsmA* homologues (e.g., *P. fluorescens* CHA0, see Section 2) or even three *rsmA* homologues (Figures 6 and 7).

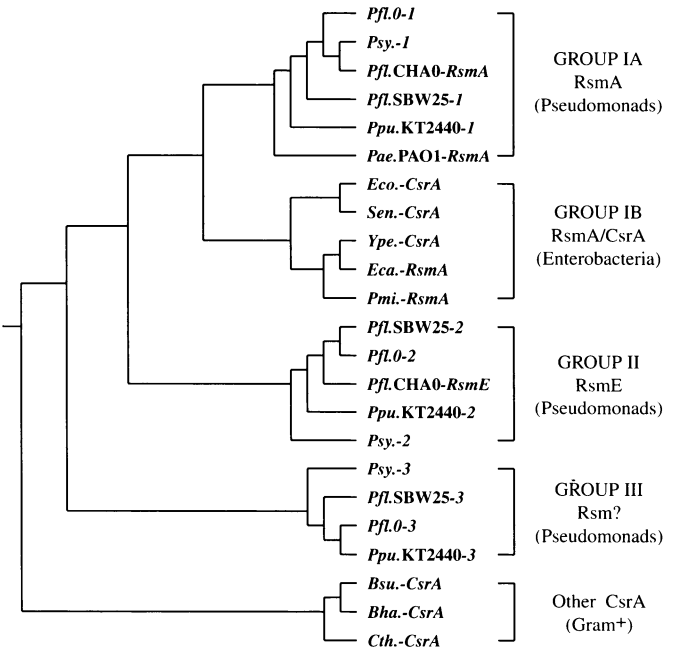

Figure 6. Dendrogram of RsmA-like proteins of some pseudomonads, enterobacteria and gram-positive bacteria, obtained by CLUSTAL W analysis[46]. The genomes of various pseudomonads reveal the presence of up to three paralogues of the global regulator RsmA/CsrA. These putative regulators have only been characterized in *P. fluorescens* strain CHA0 and *P. aeruginosa* PAO1, and in the enterobacteria. However, RsmA/CsrA homologues are found in many other eubacteria. Abbreviations: *Pfl.* CHA0, *P. fluorescens* CHA0; *Pfl.*0, *P. fluorescens* Pf0-1; *Pfl.*SBW25, *P. fluorescens* SBW25; *Psy, P. syringae* pv. *syringae; Ppu.*KT2440, *P. putida* KT2440; *Pae.*PAO1, *P. aeruginosa* PAO1; *Bsu, Bacillus subtilis; Bha., Bacillus halodurans; Cth., Clostridum thermocellum; Eco, E. coli; Sen., S. enterica; Pmi., Proteus mirabilis; Eca., E. carotovora; Ype., Y. pestis.*

GROUP Ia, identity with *E. coli* CsrA: 75%–85%

```
                        :    :    *:*:**:::*   *
  KH consensus          I I  f    VIGKKGffIR L f
CHA0-RsmA MLILTRRCAESLIIGDGEITVTVLGVKGNQVRIGVNAPKEVAVHREEIYLRIKKEKDEEPSH
PAO1      MLILTRRVGETLMVGD-DVTVTVLGVKGNQVRIGVNAPKEVAVHREEIYQRIQKEKDQEPNH
Psyr-1    MLILTRRCAESLIIGDGEITVTVLGVKGNQVRIGVNAPKEVAVHREEIYLRIKKEKDEEPSH
Pf01-1    MLILTRRCAESLIIGDGEITVTVLGVKGNQVRIGVNAPKEVAVHREEIYLRIKKEKDEEPSH
KT2440-1  MLILTRRCAESLIIGDGEITVTVLGVKGNQVRIGVSAPKEVAVHREEIYLRIKKEKDEEPSL
SBW25-1   MLILTCRCAESVILGDGEITVTVLGVKGNQVRIGVNAPKEVAVRREKIDLRVNKEKAPSX--
          ***** * .*::::**.::**************.*******:**:*  *::***  .
EcoCsrA   MLILTRRVGETLMIGD-EVTVTVLGVKGNQVRIGVNAPKEVSVHREEIYQRIQAEKSQQSSY
```

GROUP II, identity with *E. coli* CsrA: 69%–77%

```
                      * *  :    ::*::*:::*   :
 KH consensus         I I  f    VIGKKGffIR L f
SBW25-2   MLILTRKVGESINIGDDITITILGVSGQQVRIGINAPKDVAVHREEIYQRIQAGLTAPDKNQ--
CHA0-RsmE MLILTRKVGESINIGDDITITILGVSGQQVRIGINAPKDVAVHREEIYQRIQAGLTAPDKRETP
Pf01-2    MLILTRKVGESINIGDDITITILGVSGQQVRIGINAPKNVAVHREEIYQRIQAGLTAPDKPQ--
Psyr-2    MLILTRKVGESINIGDEITVTILGVQGLQVRLGINAPKNVSVHREEIYKRIQAELAPNQDPQ--
KT2440-2  MLILTRKVGESIVINDDIKVTILGVKGMQVRIGIDAPKDVQVHREEIFKRIQAGSPAPEKHE--
          ************ *.*:*.:*****.* ***:**:***:* ******::****  .. :.
```

GROUP III, identity with *E. coli* CsrA: 45%–58%

```
                      : *  :    ::   .. ::
 KH consensus         I I  f    VIGKKGffIR L f
Psyr-3    MLVLTRDIGETFSIGDDITVQILGVNGNQVRLGISAPKDIKVHRAEVYKRIANKLSQQAAQT
SBW25-3   MLVLSRAVGEVISIGDDIAVHILELNGSQVKFGVEAPNGVHVHRAEVYQKIRDRLGATTHPV
Pf01-3    MLVLSRVVGELISIGDNITLRVLAVNGSSVRFGVEAPEQVGVHRAEVYERIQRKQASGKGR-
KT2440-3  MLVIGREVGEVIVIGDDIRIMVVETRDGVVRFGVAAPREVPVHRAEVYKRIKASKQSKARG-
          ***: * :** : ***:* : ::  ... *::*: **. : *******::*
```

Figure 7. Alignment of RsmA homologues from some pseudomonads. Groups Ia, II and III (Figure 6) are aligned with CLUSTAL W[46]. As a reference for each group, minimum and maximum identities with *E. coli* CsrA are indicated. Part of the RNA-binding KH consensus motif is indicated on top of each alignment, where a lowercase f indicates a polar residue[11,26]. *, identical residues in all sequences; :, conserved substitutions; ., semi-conserved substitutions. Abbreviations: CHA0, *P. fluorescens* CHA0; PAO1, *P. aeruginosa* PAO1; Psyr, *P. syringae* pv *syringae*; Pf01, *P. fluorescens* Pf0-1; KT2440, *P. putida* KT2440; SBW25, *P. fluorescens* SBW25; Eco, *E. coli*.

The genetic contexts of *rsmE* and the homologues of the third group seem to be more variable between different species than is the *rsmA* neighbourhood. Alignment of RsmA-like homologues from pseudomonads, enterobacteria and some gram-positive bacteria using CLUSTAL W[46] and the resulting dendrogram show that the RsmA group clusters together with the RsmA/CsrA homologues of the enterobacteria (groups Ia and Ib; Figure 6). RsmE homologues, and the homologues of the third group cluster into distinct groups (groups II and III, respectively; Figure 6), suggesting that they are less closely related to the RsmA/CsrA group of enterobacteria (Figures 6 and 7). The biological significance of having two or more similar post-transcriptional regulatory

proteins and multiple riboregulators is still unclear, but may favour specificity and sequential target gene expression.

In conclusion, modulating the rate of translation of target genes by using antagonist riboregulators may allow economical utilization of metabolic resources, especially when some become limiting, as at the onset of stationary phase[50]. Small, potentially short-lived riboregulators controlling the expression of a relatively large set of genes at the translational level may facilitate rapid responses to changing environmental conditions.

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9

TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEMS: A KEY TO THE ADAPTATIVE POTENTIAL OF *PSEUDOMONAS AERUGINOSA*

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1. INTRODUCTION

The survival of bacteria depends on constant sensing of their environment and adapting to changes, these processes require signal transduction across biological membranes. A major and complex mechanism of signal transduction, widespread in bacteria, is the so-called two-component system (TCS) utilizing phosphorylation as a means of information transfer.

From the beginning of our understanding of TCS in the mid-1980s to the present, a huge amount of data and examples have been uncovered. These systems are widespread existing not only in nearly all prokaryotes and many Archae, but also in eukaryotes such as plants, fungi and yeasts[83]. Many important advances in the understanding of the mechanisms involved have occured; despite this, a great deal remains to be learnt. Relatively few TCSs have been studied in depth. Those which have been studied form a reservoir of information about how these systems function.

Starvation for phosphate or nitrogen, responses to oxygen limitation, and adaptation to new carbon and nitrogen sources are but a few of the environmental changes that cells overcome with modified cellular physiology

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mediated by TCS. TCSs are central to much of the cellular physiology that results from alterations in the environment. Pathogenesis also requires two-component modification of cellular physiology as well. There is little doubt that cells sense when they need to express virulence factors, but, in most cases, what is sensed remains obscure. It is unlikely that any pathogen can survive in the varied and changing environments of the human body without involving at least one two-component pathway.

Pseudomonas aeruginosa is an ubiquitous environmental bacterium that is one of the top three causes of opportunistic human infections. It is a versatile gram-negative bacterium that grows in soil and aquatic habitats as well as on plant and animal tissues. It forms biofilms on wet surfaces such as rock and soil. The emergence of *P. aeruginosa* as a major opportunistic pathogen may be a consequence of its remarkable capacity to adapt to many hosts. Indeed, this opportunistic pathogen proliferates within hosts as varied as plants, insects, nematodes and mammals and interacts with amebae and yeasts as well. *P. aeruginosa* is a significant source of bacteremia in burn victims, urinary tract infections in catheterized patients and hospital-acquired pneumonia in patients on respirators. It is also the predominant cause of morbidity and mortality in cystic fibrosis (CF) patients, whose abnormal airway allows for long-term colonization of the lungs by *P. aeruginosa*. These infections are difficult to eradicate, in part because of the natural resistance of the bacterium to antibiotics, and often lead to pulmonary failure and death.

The complete genome sequence (6.3 Mbp) has been published[68]. The published analysis predicts that 8.4 % of *P. aeruginosa* genes are involved in regulation, which is the highest proportion of predicted regulatory genes observed among sequenced bacterial genomes. For example, characteristic motifs of regulatory proteins are found in 5.8 % of *Escherichia coli* genes and 5.3% of *Bacillus subtilis* genes, but only in 3 % of genes in *Mycobacterium tuberculosis*, a highly specialized pathogen with a comparable genome size. The large genome size and genetic complexity of *P. aeruginosa* reflect evolutionary adaptations permitting it to thrive in diverse ecological niches and analysis of the complete genome reveals clues regarding this versatility.

The most striking over-representation is in two-component regulatory systems. There is an extraordinary number of putative TCS proteins with 63 sensors and 64 response regulators (RR)[63, 68]. To date, this is the second largest number of signal transduction proteins of any sequenced bacterium when adjusted for genome size (127/6264 nt), just after *Caulobacter crescentus* (105/4017 nt)[49]. These numerous regulatory proteins presumably modulate the diverse genetic and biochemical capacities of this bacterium in adapting to changing environmental conditions.

In this review, we will define general principles of TCS structural organization followed by the description of some newly discovered domains in this

family of regulatory systems. Along this line, we will examine the complexity of the *P. aeruginosa* TCSs and focus on some particularly interesting cases. The integration of TCSs in cellular physiology and global regulatory networks will then be discussed.

2. STRUCTURE–FUNCTION STUDIES OF TWO-COMPONENT SIGNAL TRANSDUCTION SYSTEMS: GENERAL PRINCIPLES

A typical TCS includes a histidine kinase (HK) and a partner RR. Sensor kinases (or HKs) usually possess two domains: an input domain, which monitors environmental stimuli, and a transmitter domain, which autophosphorylates following stimulus detection. The input domain varies in length and amino acid sequence from one HK to another. The diversity of sensor domains probably reflects the wide range of environmental stimuli that elicit specific regulatory responses in bacterial cells. By contrast, the transmitter domain shows high sequence conservation. It contains an invariant histidine residue that is phosphorylated in an ATP-dependent manner and short stretches of conserved amino acids, in particular two glycine-rich motifs involved in ATP binding (the NG1FG2 motif; Figure 1). A classical RR contains a conserved amino-terminally located receiver domain that is phosphorylated by the sensor kinase at a strictly conserved aspartate residue, leading to activation of the carboxy-terminal effector or output domain. More complex systems exist and have been defined as phosphorelays (for a review, see ref. [59]) (Figure 1). Phosphorelays differ from TCSs by having a more complicated signal transduction pathway using additional regulator and phosphotransferase domains. In the sporulation phosphorelay of *B. subtilis*, for example, these domains are on individual proteins but in many systems these domains are associated with the kinase in a single polydomain protein. The additional domains/components offer more targets for regulation of the pathway: sensing more input signals or regulating different targets and adding some levels where phosphatases, which regulate phosphate flow by dephosphorylating the receiver domain, can act. When the phosphorelay signal transduction pathway was discovered, it was suggested that increased complexity over a TCS may reflect the need to integrate both positive and negative signals into the output of the pathway[23].

2.1. Histidine Kinase Domains

2.1.1. Sites of Phosphorylation and Dimerization

Phosphorylation-Induced Conformational Changes. Classical sensory kinases are composed of a transmitter domain preceded by an amino-terminal

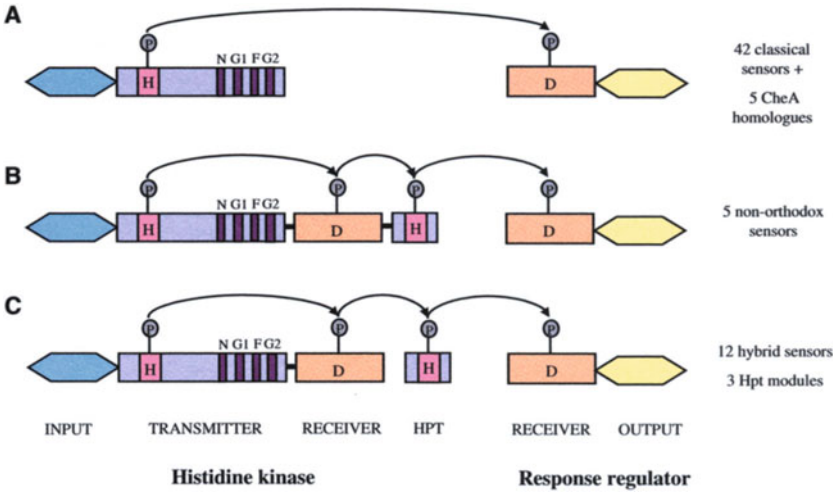

Figure 1. Representation of phosphorelay cascade between HK sensor and RR of TCSs in *P. aeruginosa*. A a classical system, B a non-orthodox system and C a hybrid system involving a histidine phosphotransfer (Hpt) module. H is the conserved histidine residue phosphorylated upon stimulus detection, D is the invariant aspartate residue receiving the phosphate from the transmitter or Hpt domain and NG1FG2 is the conserved ATP binding site.

input domain. These kinases are identified by unique signature sequences called H, N, G1, F and G2 boxes[59, 71]. In classical kinases, the H box-containing region is directly linked to the region that contains the other four conserved boxes (this is not the case for the HK of the CheA family). The kinase domain is 350 amino acids in length and is responsible for binding ATP and directing kinase transphosphorylation. The conserved histidine substrate is the central feature in the H box, whereas the N, G1, F and G2 boxes define nucleotide binding cleft and catalytic activity[1]. In most HKs, the H box is part of the dimerization domain. The N, G1, F and G2 boxes are usually contiguous, but the spacing between these motifs is variable. The folding of the HK catalytic domain is different from the folding of Ser/Thr/Tyr kinase, suggesting that the mechanism of action of this histidine may be different from that of other previously characterised prokaryotic and eukaryotic kinases[73].

Most of the sensors are active in a dimeric form. It has been shown that phosphorylation of the active-site histidine occurs *in trans* within a dimer[52, 70] and the histidine of one subunit is phosphorylated by the other subunit. Kramer and Weiss[40] worked on the HK NtrB of *E. coli* and proposed that the function of the sensor domain could be to change the relative orientation of the transmitter domains toward each other such that, in the active conformation, the

catalytic domain of one subunit would be able to interact with the H domain of the other subunit, leading to phosphorylation of the active-site histidine.

The input domain varies in length and amino acid sequence from one HK to another, conferring specificity for different stimuli proteins. Its structure depends on the signal sensed by the TCS.

2.1.2. Transmembrane Domains

Many HK sensors are localized in the inner membrane due to the presence of a transmembrane (TM) hydrophobic domain in their amino acids sequence. The number of the TM segments defining the TM domain is variable.

2.1.3. Additional Signatures

Sequence analysis of sensor modules and response modules encoded in complete bacterial and archaeal genomes revealed complex domain architectures for many of them (for a review. see ref. [29]). All these domains are widely represented in bacteria. In contrast, these novel signaling domains are much less abundant in bacterial parasites and in *Archaea*. Domain architecture has proven particularly informative for analyzing multidomain proteins involved in signal transduction. In HK, several new domains have been described and we will briefly describe them in this section.

2.1.3.1. HAMP Linker Domains. Sequence comparisons and mutational analysis of sensor kinases and methyl-accepting proteins (MCP) allowed the identification of a common structural element hypothesized to transmit signals between the external input domain and cytoplasmic transmitter domain[7]. This element was called the HAMP (*h*istidine kinase, *a*denyl cyclase, *M*CP and *p*hosphatase) linker, or the P-type linker. The HAMP linker comprises approximately 50 amino acids distal from the second TM segment. This region includes two stretches of hydrophobic amino acids in a heptameric arrangement, with a phased spacing of hydrophobic residues characteristic of amphipathic α-helices. The complete HAMP linker is predicted to consist of these two amphipathic α-helices linked by an unstructured domain. The general model depicts the HAMP linker as a rigid pushrod connecting the input domain to the output domain[26]. Ligand binding to the periplasmic input domain causes a small (1–2 Å) sliding displacement between the second TM helix in the sensor dimer[57]. This displacement results in an identical displacement at the distal end of the HAMP linker, which abuts the output domain. Recently, mutagenesis analysis of the *E. coli* nitrate sensor NarX revealed that structure rather than sequence is the key feature of the HAMP linker[6]. Appleman and Stewart[6] showed that the HAMP linker is a negative effector of the sensor output activity and that the signal from the periplasmic domain relieves the negative interactions between the HAMP linker and the

output domain. In any case, this domain, especially the second amphipathic helix, has a critical role in signal transduction.

2.1.3.2. PAS Domain. PAS is an acronym formed from the names of the proteins in which imperfect repeat sequences were first recognized: the *Drosophila* period clock protein (*P*AR), vertebrate aryl hydrocarbon receptor nuclear translocator (*A*RNT), and *Drosophila* single-minded protein (*S*IM). PAS domain comprises a region of approximately 100–120 amino acids (for a review, see ref. [75]). The core of the PAS domain structure is made by three predicted β-strands that form a β-sheet. This domain is found predominantly in proteins that are involved directly or indirectly in signal transduction. Among *Bacteria* and *Archaea*, PAS domain are found almost exclusively in sensors of two-component regulatory systems associated with soluble sensor or in the cytoplasmic part of a TM sensor. The cytoplasmic localization of the PAS domain suggests that they sense changes in the intracellular environment. Where a function is known, PAS domains mostly sense redox potential, oxygen, cellular energy or light. The number of PAS domains per protein may vary from one to six, in such a way that one sensor protein can respond to multiple input signals, each activating a specialized PAS domain.

2.1.3.3. GAF Domain. GAF domains have been described by Aravind and Ponting[8]. They initially identified this domain in c*G*MP-specific and stimulated phosphodiesterases, *Anabaena a*denylate cyclases and *E. coli F*hlA protein. One well-characterized function of the GAF domains is cGMP-binding[14]. It is suggested that GAF domains may control cGMP-dependent signaling events. GAF domains may regulate allosteric-dependent catalytic activities via the binding of ligands, such as nucleotides and small molecules. These catalytic activities might also include HK ATPases, for example. Even though there is no sequence similarity between PAS and GAF domains, they show close structures similarity[47].

2.2. Response Regulator Domains

Response regulators are composed of an N-terminal domain or receiver domain, dedicated to receiving the phosphate from the sensor and a C-terminal domain or output domain that can be a transcriptional regulatory domain or have a catalytic activity. The output domains of RRs can be classified into different subfamilies on the basis of whether they are similar to the *E. coli* OmpR, NarL or NtrC output domains. Analysis of RR sequences revealed an even greater diversity. In addition to well-known RR, bacterial genomes were found to encode a variety of RR with unusual

domain organization, featuring still poorly characterized domains, such as those described below.

2.2.1. The OmpR Family

The carboxy terminal domains of the OmpR family contain an amino terminal four-stranded β sheet, a central three-helical bundle and a carboxy-terminal β hairpin[55]. Residues that form the hydrophobic core are mostly conserved throughout the family. The DNA binding motif is a winged helix–turn–helix (wHTH). The turn in the HTH of the *E. coli* OmpR is unusually long (10 amino acids compared to the more usual 3–4 in other HTH proteins). Phosphorylation drives a conformational change in OmpR that promotes interaction between OmpR molecules at the adjacent sites and also with the RNA polymerase, leading to the activation or repression of transcription[37].

2.2.2. The NarL Family

The structure of the *E. coli* RR NarL was solved several years ago[9]. In spite of the great folding similarity between the C-terminal domain of NarL and other HTH proteins, it cannot interact directly with DNA as they do, because the DNA binding site is blocked by the N-terminal unphosphorylated domain. It appears that the phosphorylation-induced activation must involve an opening of the N- and C-terminal domains to liberate the DNA binding domain[9].

2.2.3. The NtrC Family

The most structurally and functionally complex RR family is that represented by the nitrogen regulatory protein NtrC, a transcriptional enhancer that activates σ^{54}-holoenzyme form of RNA polymerase. The effector domain of the NtrC-family proteins consists of two domains: an ATPase domain and an HTH DNA binding domain[56]. The ATPase domain is similar to those found in a large family of protein described as AAA+ proteins. These proteins are chaperon-like ATPases often acting as molecular machines involved in the formation and remodeling of protein and protein–nucleic acid complexes[60]. It has been shown that NtrC dimers, capable of binding DNA, oligomerize into octamers upon phosphorylation[88]. Oligomerization of the regulator stimulates ATP hydrolysis which provides energy for open complex formation and activation of transcription[82]. The transcriptional activator is usually bound at least 100 bp upstream of the promoter site, and DNA looping is required for the activator to contact the closed complex. The crystal structure of the phosphorylated NtrC confirms that phosphorylation alters the molecular surface of the receiver domain[38]. The phosphorylation of the receiver domain permits the exposition of a hydrophobic surface proposed to allow transmission of the signal to the effector domain. A recent review describes the domain architectures of σ^{54}-dependent transcriptional activators[69].

2.2.4. Additional Signatures

Five types of newly described domains are present in the C-terminus part of atypical regulators. Four of them have putative enzymatic activity[29], the fifth one is predicted to share receptor activity meaning that the regulators bearing this domain have detector and effector functions.

2.2.4.1. The EAL Domain. It was originally described in a study of BvgR in *Bordetella pertussis*. This regulatory protein is involved in regulation of the major virulence factors, together with the TCS BvgAS[76]. This domain is also found in diverse bacterial signaling proteins, and is called EAL after the invariant residues found within the domain. The EAL domain is a good candidate for a diguanylate phosphodiesterase function. It contains many conserved acidic residues that could participate in metal binding and might form the phosphodiesterase active site.

2.2.4.2. HD and HD-GYP Domains. The HD superfamily, designated after the principal conserved residues implicated in metal binding and catalysis, represents a superfamily of metal-dependent phosphohydrolases. The activity of the HD-GYP domains is less characterized. They may have a phosphatase or phosphodiesterase activity. The highly conserved sequences suggest a high substrate specificity and they may be involved in the metabolism of cyclic diguanylate or in dephosphorylation of a phosphotransfer domain.

2.2.4.3. The GGDEF Domain. The GGDEF domain was first discovered in the RR PleD that control cell differentiation in the swarmer-to-stalked cell transition in *C. crescentus*[34]. This domain is found linked to a wide range of other domains in a variety of bacteria. Its function is still unknown, however it has been shown to be homologous to the adenylyl cyclase catalytic domain. If the GGDEF and EAL domains indeed function as a diguanylate cyclase and a c-diGMP phosphodiesterase, respectively, c-diGMP could emerge as a major cell regulator in bacteria.

2.2.4.4. The CACHE Domain. CACHE domains are particularly widespread in bacteria. They were first described as signaling domains common to animal Ca^{2+} channel subunits and a class of prokaryotic chemotaxis receptors[3]. Most of the proteins having a CACHE domain are receptors that bind small molecules via this domain and convert this signal into diverse outputs depending on their intracellular effector domains. This domain contains three predicted β-strands that could form a sheet analogous to that present in the core of the PAS domain structure.

2.2.4.5. PP2C Domain. This domain is described as a sigma factor phosphatase.

3. ANALYSIS OF THE TWO-COMPONENT REGULATORY SYSTEMS IN *P. AERUGINOSA*

3.1. Number of Putative TCSs in the Whole Genome, Genetic Organization and Sequence Analysis

As already mentioned, a compilation of the TCS retrieved from the completed sequence was published, with the aim to depict the structure and organization of the two-component phosphorelays in *P. aeruginosa*. This search led to the identification of putative genes encoding 63 HK sensors and 64 RRs[63]. A new treatment of the genomic sequence allows us to increase the number of RRs present in the whole genome up to 66 regulators (I. Ventre, unpublished data).

Genes encoding a cognate sensor–regulator pair involved in a specific signaling pathway are usually located close to each other or even belong to the same transcriptional unit. Among the 14 TCS pairs previously characterized (Table 1), only the genes encoding GacS and GacA are not close to each other.

Table 1. Known two-component regulatory systems in *P. aeruginosa*.

Sensor	Response regulator	Physiological process	References
Two-component pairs			
AlgZ/FimS	AlgR	Alginate production	[22], [89]
ChpA	ChpB	Chemotaxis and virulence factors production	[46]
CrbA	CrbB	Catabolism of natural substrate	[53]
ExaD	ExaC	Ethanol oxidizing system	[30]
FleS	FleR	Adhesion	[62]
GacS	GacA	Virulence factors production	[61], [74]
KinB	AlgB	Alginate production	[87]
NarX	NarL	Nitrate acquisition	[41]
PfeS	PfeR	Iron acquisition	[20]
PhoB	PhoR	Phosphate level	[5]
PhoQ	PhoP	OprH production, polymyxin and aminoglucosides resistance	[44], [45]
PilS	PilR	Type IV pili production	[35]
PirS	PirR	Iron acquisition	[78]
PprA	PprB	Membrane permeability	[80]
Partially characterized systems			
?	AgmR	Glycerol metabolism	[65]
?	GltR	Glucose uptake	[64]
?	PilG, PilH	Chemotaxis/pili production	[18]
?	TrpO	Tryptophane biosynthesis	[33]
?	WspR	Autoaggregation	[16]

Among the genes encoding two-component transducers, 50 sets are located in close proximity whereas the remainder (12 sensor genes and 8 RR genes) are not. For clarity in the text, we will discuss these particular genetic organizations along with their structural characteristics. We hypothesize that the proteins encoded by orphan genes could function with more than one partner and, as a result, they might participate in cross-communication.

Signaling proteins are annotated in sequence databases (http://www.pseudomonas.com) as "sensor protein" or "response regulator." Because of the emerging complex picture of prokaryotic signal transduction systems, we searched in the *P. aeruginosa* genome for the presence of the various domains previously described (Sections 2.1.3 and 2.2.4). Comparative sequence analyses can provide initial clues to the importance of particular structural features in transmitter and receiver modules. Only a few proteins containing these domains have been studied experimentally and their exact biochemical functions remain obscure.

Therefore, we searched for the presence of the various domains previously described in Section 2, by using the Smart (http://smart.embl.heidelberg.de/) and the CDD programs (http://www.ncbi.nlm.gov/Structure/cdd/wrpbs.cgi). TM domains were searched in all the HKs of *P. aeruginosa* by using the TMPRED program (http://www.ch.emmet.org/software/TMPRED_form.html).

A compilation of our results is presented in Tables 2 and 3. The abundance of various domains was unexpected. Here, we briefly review the diversity and the complexity of newly discovered signaling domains in the TCS of *P. aeruginosa* and discuss their emerging complex picture.

3.2. Sub-Families of Sensory Kinases

3.2.1. Classical HKs

Sequence analysis of the 63 potential HKs identified in the genome of *P. aeruginosa* revealed 42 putative classical sensory kinases (Table 2), with most of the previously identified kinases belonging to this family[63]. The *P. aeruginosa* genome contains also 5 genes encoding kinases homologous to the chemotaxis protein CheA.

A search for TM segments revealed a large diversity in the number and the organization of the TM domains of these sensors. Five of the classical HKs are predicted to be soluble. The other sensors are predicted to be localized in the inner membrane and have from 1 to 12 TM segments (Table 2). Among all these cases, we will discuss only a few of them having particular characteristics.

The FleS/FleR TCS has been demonstrated to regulate motility and adhesion to mucins by *P. aeruginosa*[62]. Richtings *et al.*[62] analyzed the hydrophobicity of FleS and concluded, as we did, that FleS might be a soluble and cytoplasmic protein.

Table 2. Domain organization of the two-component sensors of *P. aeruginosa*.

PA number	Number of TM segments	Particular domains
Classical sensors: 42 members		
PA0464 *creC – phoM*	2	HAMP
PA0600	2	PBPb – 2 PAS
PA0757	2	HAMP
PA0930 *pirS*	1	HAMP
PA1098 *fleS*	0	PAS
PA1158	1	HAMP
PA1180 *phoQ*	2	HAMP
PA1336	2	—
PA1438	2	HAMP
PA1636 *kdpD*	5	KdpD – GAF
PA1798	2	HAMP
PA1979	0	—
PA2480	3	HAMP
PA2524	3	HAMP
PA2571	1	—
PA2656	2	HAMP
PA2687 *pfeS*	2	HAMP
PA2810	2	HAMP
PA2882	0	—
PA3078	2	—
PA3191	2	HAMP
PA3206	2	HAMP
PA3878 *narX*	2	HAMP
PA4036	5	PAS
PA4102	3	HAMP
PA4117	3	GAF – phytochrome
PA4197	2	2 PAS
PA4293 *pprA*	0	GAF – 2 PAS
PA4380 *colS*	2	HAMP
PA4398	3	HAMP – PAS
PA4494	5	—
PA4546 *pilS*	6	PAS
PA4725 *crbA*	12	PAS
PA4777	2	HAMP
PA4886	2	HAMP
PA5124 *ntrB*	1	PAS
PA5165	2	—
PA5199 *envZ*	2	HAMP
PA5262 *algZ fimS*	4	—
PA5361 *phoR*	2	PAS
PA5484 *kinB*	2	HAMP—PAS
PA5512	4	—
Non-orthodox sensors: 5 members		
PA0928 *gacS*	2	HAMP – HPT

Table 2. *Continued*

PA number	Number of TM segments	Particular domains
PA3044 *bvgS*	2	PAS – HPT
PA3946 *bvgS*	2	2 PBPb – PAS – HPT
PA4112	3	CHASE – HPT
PA4982	4	HAMP – HPT
Hybrid sensors: 12 members		
PA1243	0	GAF – PAS
PA1396	5	—
PA1611	2	HAMP
PA1976	0	2 PAS
PA1992	0	—
PA2177	0	2 PAS
PA2583 *bvgS*	2	2 PBPb
PA2824	1	—
PA3271	12	PAS
PA3462	7	—
PA3974	6	—
PA4856	7	—
CheA family: 4 members		
PA0413 *chpA*	PA0178 *cheA2*	PA1458 *cheA* PA3704 *wspE*

PA numbers represent annotated sequences from the *Pseudomonas* web site: http://www.pseudomonas.com. All the two-component sequence genes were analyzed using the TMPRED program, to determine the TM domain, and the Smart and CDD program to search for particular domains. The particular domains were defined as follows: HAMP (*h*istidine kinase, *a*denyl cyclase, *M*CP and *p*hosphatase), PAS (*P*AR, *A*RNt, *S*IM), GAF (c*G*MP-specific and stimulated phosphodiesterases, *Anabaena a*denylate cyclases and *E. coli F*hlA protein), HPT (*h*istidine *p*hospho*t*ransfer), PBPb (*b*acterial *p*eriplasmic substrate-*b*inding *p*rotein) and CHASE (*c*yclases, *h*istidine kinases *a*ssociated *s*ensing *e*xtracellular).

Among the *P. aeruginosa* TCSs, the example of PilRS is well documented. The HK PilS (PA4546) and its RR PilR (PA4547) regulate transcription of the pilin gene *pilA*[35]. PilS is an inner membrane protein with a molecular weight of 59 kDa[11]. Ethier and Boyd[25] showed that the N-terminal with 174 amino acids of PilS is highly hydrophobic and forms 6 TM helices. The central domain or linker domain (residues 177–296) is cytosolic and has weak homology to PAS domain. The C-terminal transmitter domain (residues 297–530) contains all the conserved amino acids characteristic of sensor kinases. PilS seems to be localized at the pole of the cell at which PilA is assembled into type IV pili[10].

CrbA (catabolic regulation, PA4725) is a classical HK which functions with CrbB (PA4726), an RR belonging to the NtrC family[53]. This TCS controls several specific metabolic pathways and modulates the catabolism of various

Table 3. Two-component RRs of *P. aeruginosa*.

OmpR family: 24 members		
PA0463 *creB*	PA0756	PA0929 *pirR*
PA1157	PA1179 *phoP*	PA1437
PA1637 *kdpE*	PA1799	PA2479
PA2523	PA2657	PA2686 *pfeR*
PA2809	PA3077	PA3192 *gltR*
PA3204	PA4032	PA4101
PA4381 *colR*	PA4776	PA4885 *irlR*
PA4983 *dmsR*	PA5200 *ompR*	PA5360 *phoB*
NarL family: 14 members		
PA0034 *trpO*	PA0601	PA1397
PA1978 *agmR*	PA1980	PA2586 *gacA*
PA3045 *bvgA*	PA3604	PA3714
PA3879 *narL*	PA3948 *bvgA*	PA4080 *rcsB*
PA4196	PA4296 *pprB*	
NtrC family: 8 members		
PA1099 *fleR*	PA1335	PA4547 *pilR*
PA4726 *cbrB*	PA5125 *ntrC*	PA5166
PA5483 *algB*	PA5511	
Atypical RRs: 11 members		
PA2572	PA2798	PA2881
PA3346	PA3947	PA4396
PA4493	PA4781	PA4843
PA5261 *algR*	PA5364	
CheB family: 4 members		
PA0173 *cheB2*	PA0414 *chpB*	PA1459 *cheB*
PA3703 *wspF*		
CheY family: 5 members		
PA0179 *cheY2*	PA0408 *pilG*	PA0409 *pilH*
PA1456 *cheY*	PA3702 *wspR*	

natural substrates in response to different C/N ratios. CrbA is a 490 amino acid protein and presents a very unusual feature with as many as 12 possible TM helices. This membrane domain shows about 20 percent identity with sodium/solute symporters. Even though, it has not been further analyzed, this protein seems to be a hybrid of a permease-like domain and a classical transmitter domain of HK. It would be interesting to know whether the permease domain is just a sensor domain or if it could also be an active pump whose function might serve as initial signal transmission.

FimS/AlgZ (PA5262) is the cognate sensor of AlgR (PA5261), an atypical RR (see below)[84]. We predicted that FimS has four TM segments, but lacks the conserved residues implicated in nucleotide binding, suggesting that FimS

may be incapable of autophosphorylation. AlgR was shown to activate transcription from the *algD* gene promoter, the product of which is a key enzyme in alginate biosynthesis[22]. More recently, it was shown that AlgR and FimS are also required for controlling twitching motility in *P. aeruginosa*[84]. Phosphorylation of AlgR is not required for *algD* regulation[43]. In contrast, phosphorylation of AlgR is essential for type-IV mediated twitching motility[85]. Thus, FimS and AlgR appear to function differently in regulating twitching motility and alginate production, although in other respects there is good evidence that they constitute a true TCS. Because FimS lacks the crucial residues required for autophosphorylation activity, it is possible that FimS blocks or reverses AlgR phosphorylation[85].

Recently, a new TCS has been identified in *P. aeruginosa* by Wang *et al.*[80]. PprA (PA4293) is a classical sensor lacking a signal sequence and TM domain, so it is likely to be a cytoplasmic protein. They identified in the N-terminal part of PprA a PAS domain; additionally we identified a GAF domain. Possibly, this protein could sense multiple kinds of stimuli. The cognate RR is PprB (PA4296) belonging to the NarL family. This TCS is involved in regulating the outer membrane permeability of *P. aeruginosa*. Usually, genes encoding a functional pair of sensor and RR are organized in tandem. In this respect, the genetic organization of the PprA/PprB TCS is quite surprising, the two genes *pprA* and *pprB* seem to be transcribed from divergent promoters, and they are separated by two other genes encoding proteins of unknown function (http://www.pseudomonas.com). It would be very interesting to know if these two ORFs have any activity in the two-component PprA/PprB transduction pathway.

3.2.2. Non-Orthodox Sensors

This class of sensors corresponds to HKs that possess an additional receiver domain adjacent to the transmitter domain. This receiver domain is similar to those of RRs and is linked to a histidine phosphotransfer module (HPT domain) (Figure 1). These sensors are involved in phosphorelays where the signal is transmitted in three phosphotransfer steps. *P. aeruginosa* genome could encode five non-orthodox kinases[63]. The HPT domain is small, approximately 120 residues, and contains a histidine residue that mediates phosphotransfer. The 3D-structure of the HPT domain of the non-orthodox sensor ArcB of *E. coli* have been solved[36]. It is essentially an α-helical structure, consisting of six helices forming a kidney-shaped molecule. Although sequence comparisons between different HPT domains show less than 20% sequence identity, conserved residues important for their structure and function suggest that all HPT domains share a common structural motif. This domain does not exhibit by itself any kinase or phosphatase activity.

We analyzed the TM composition of the five non-orthodox sensors of *P. aeruginosa*. All these kinases are predicted to be located in the inner membrane with 2–4 TM segments depending on the protein (Table 2).

The sensor GacS (PA0928), involved in regulation of virulence, is a non-orthodox sensor. This kinase was shown to work with the RR GacA. In *P. aeruginosa*, GacA is a global regulator of virulence factors[61]. The genes encoding GacA and GacS are not close to each other in PAO1 (*gacA* = PA2586). Moreover, GacA is close to a hybrid HK (see below). GacS possesses a HAMP linker between its TM domain and the transmitter domain.

In *B. pertussis*, the couple BvgS/BvgA controls many virulence factors. In *P. aeruginosa* there are three sensors presenting some homologies with BvgS, two non-orthodox and one hybrid. PA3044 is one of these and is tandemly organized with a BvgA homolog, PA3045, shown in *B. pertussis* to be the cognate RR of BvgS.

PA3946 is also a BvgS homolog located near another BvgA homolog, PA3948. However, between these two genes, there is a gene encoding an atypical RR (see below). We can suppose that the non-orthodox sensor PA3946 transmits the signal from its second transmitter domain onto the two RRs PA3947 and PA3948.

These two BvgS homologs have two predicted TM segments which form a long periplasmic loop. On this loop, two PBPb domains (*b*acterial *p*eriplasmic substrate-*b*inding protein) are found[72]. These domains may correspond to the input domain of the kinase. Moreover, both BvgS-like HKs have a PAS domain located in the cytoplasmic part of the protein, just before the transmitter domain.

PA4112 is an orphan non-orthodox sensor. It is a very particular protein in that it is the only protein in the PAO1 genome to have a CHASE domain[48]. This domain is 200–230 residues long, and is always found in periplasmic or extracellular regions of sensor proteins[4]. The CHASE domain is proposed to be a receptor for low molecular weight molecules and could be involved in sensing specific stimuli for the developmental program of an organism.

3.2.3. Hybrid Sensors and Hpt Modules

Twelve hybrid sensor HKs have been proposed in the genome of *P. aeruginosa*. Hybrid sensors, like non-orthodox sensors, have a transmitter and a receiver domain, but they lack the second transmitter domain required for transferring the phosphate group onto the receiver domain of the RR. This type of sensor needs a separate HPT relay. Only three genes encoding putative HPT modules have been found in the genome of PAO1[63]. Similar to the classical kinases, a large diversity in the TM domain organization exists among the hybrid sensors (Table 2). Four of the hybrid sensors seem to be soluble lacking any predicted TM segment. The others are located in the inner membrane, having from 1 to 12 TM segments.

Nine of the eleven genes encoding hybrid sensors are orphan on the genome of PAO1. This observation allows us to discuss the notion of the evolution of TCSs (for a review, see ref. [39]). There are two competing models

for the evolution of novel TCS: The recruitment model and the coevolution model. The recruitment model suggests that novel TCS evolve through gene duplication of one component, which then use components from heterologous systems to yield a new specificity. The coevolution model suggests that novel TCS evolve by global gene duplication of all their components and subsequent differentiation. The two models are not exclusive, but the number of orphan hybrid kinases in *P. aeruginosa* genome argues in favor of the recruitment model.

The HKs of *P. aeruginosa* have other peculiarities. Some of them have several specific domains described above: PAS domain, GAF domain and HAMP linker (Table 2).

We predicted that PA3271 has 12 TM segments, which is very unusual. It is the second sensor in *P. aeruginosa* to present such a characteristic (with CrbA) and could form a sodium/solute symporter structure.

PA2583 is the third BvgS homolog identified in the PAO1 genome. This protein possesses two predicted TM segments delimiting a long periplasmic loop containing a PBPb domain, such as the two other BvgS homologs described. However, as a hybrid sensor, it lacks the second transmitter domain predicted to transfer the phosphoryl group on a BvgA-like RR. Even though the gene encoding PA2583 appears to be an orphan, it is located close to the gene encoding GacA (PA2586). This genetic organization raises an interesting question: Could PA2583 transmit the phosphate on GacA via an HPT under specific condition(s)?

The last special hybrid kinase we would like to focus on is PA4856. In its C-terminal part, this protein presents two tandem receiver domains. It would be interesting to know if the two receiver domains are active and if so, can transmit these phosphates onto one or two distinct RRs.

3.3. Sub-Families of Response Regulators

PAO1 possesses 66 putative RRs (11 Che-like proteins) involved in two-component signal transduction systems. Most of them have a transcriptional activity in their output domain and are classified depending on the nature of their HTH motif: OmpR family (24 members), NarL family (14 members) and NtrC family (8 members). Moreover, *P. aeruginosa* possesses 11 atypical RRs, with catalytic or unknown activity in their output domains[63]. These are all further detailed in Figure 2 and Table 3.

3.3.1. The OmpR Family

Among the 24 members of the OmpR family, only 11 of them have been characterized (Figure 2 and Table 3). All the RRs of this family are located in the chromosome of *P. aeruginosa* close to a HK sensor, except for two of them.

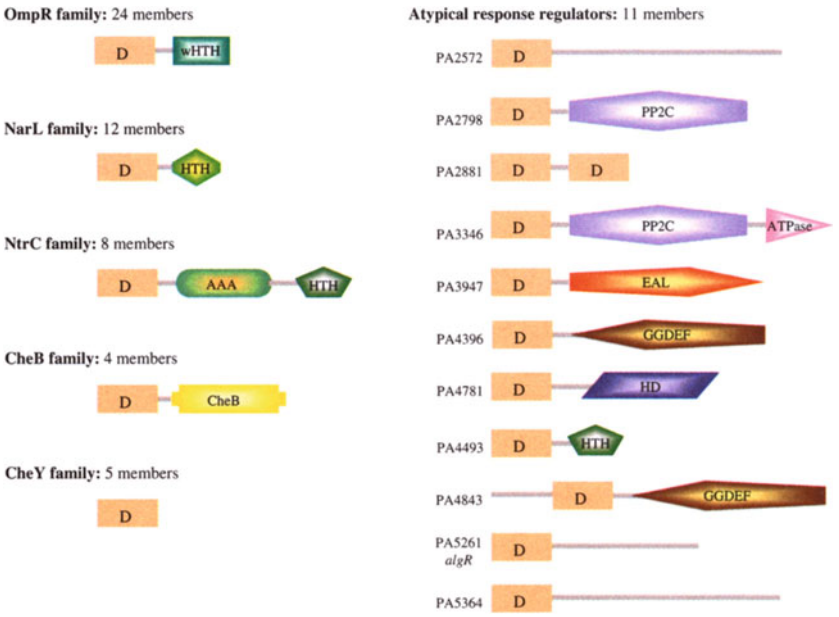

Figure 2. Representation of the domain organization of the RRs. D is the aspartate residue conserved in the receiver module. The other domains are described in the text.

PA3204 is separated from a classical sensor PA3206 by one ORF of unknown function, but the genes encoding these proteins seem to belong to the same gene cluster. PA4032 could be an orphan RR since it is separated by three ORFs from the classical sensor PA4036, and both appear to be produced from genes orientated in opposite direction on the genome.

3.3.2. The NarL Family

In this class of RRs, we can find GacA (PA2586) and two BvgA homologs (PA3045 and PA3948) (see also Section 3.2.2; Figure 2 and Table 3). GacA is known to be important for regulation of the expression of virulence factors in *P. aeruginosa* and in other bacteria.

P. aeruginosa also has a NarL homolog (PA3879) and a homolog of its cognate sensor NarX (PA3878)[41]. As in *E. coli*, NarL is involved the response to nitrate and participates in the regulation of heme biosynthesis[67].

3.3.3. The NtrC Family

It was shown recently that *P. aeruginosa* has 21 σ^{54}-dependent transcriptional activators, eight of them being RRs[69] (Figure 2 and Table 3).

FleR (PA1099) and its cognate sensor FleS (PA1098) are involved in the synthesis of flagella by *P. aeruginosa*. In this bacterium, the cascade of activation leading to production and assembly of a polar flagella is controlled by the sigma factor RpoN (σ^{54}). The fact that FleR is an RR belonging to the NtrC family is related to the role of RpoN in this control.

3.3.4. Atypical Response Regulators

Eleven atypical RRs are present in the *P. aeruginosa* genome (Figure 2 and Table 3). The effector domain of these regulators cannot be classified in any family described above (Figure 2). Half of them are encoded by orphan genes. This data is especially meaningful in respect to TCS evolution in the bacterial genome (see Section 3.2.3). Moreover, most of the atypical RRs possess additive signatures described above (Figure 2). We would like to point out two of them.

PA4493 has a C-terminal output domain homolog to the HTH domain of the NtrC family RR. However, this protein lacks the ATPase domain, and should be unable to function with the RNAP-σ^{54} to open the initiation complex.

AlgR (PA5261) is also an atypical RR. It belongs to a new class of DNA binding regulator: the LytTR family[50]. The sequence of the LytR domain is not well conserved but it has been found in proteins involved in regulation of virulence determinants in different bacteria: AgrA and LytR in *Staphylococcus aureus*[13], ComE in *Streptococcus pneumoniae* and VirR in *Clostridium perfringens*. In *P. aeruginosa*, AlgR is involved in the regulation of type-IV mediated twitching motility and in the regulation of alginate synthesis. In these two regulations, the molecular mechanism of action of AlgR seems to be different (see FimS discussion, Section 3.2.1).

4. ROLE OF TCSs IN CELLULAR PHYSIOLOGY

Bacteria live in precarious environments. Nutrient levels, acidity, temperature, osmolarity, humidity and many other conditions can change rapidly and unexpectedly. To survive, the cells must constantly monitor external conditions and adjust their structure, physiology and behavior accordingly. TCSs are sophisticated signaling systems for eliciting adaptative responses. They readily detect minute fluctuations in many chemical and physical conditions, which in turn trigger changes in gene expression that enhance survival prospects.

4.1. Sensing Nutrients and Starvations

For instance, when certain nutrients become limiting, bacterial cells can accommodate their physiology in order to use nutrients at lower concentration or alternatively, energetically less favorable nutrients. In other bacteria, it is well known that TCSs involved in such nutritional adaptation include, for example,

the NtrB–NtrC (nitrogen assimilation) and PhoR–PhoB (phosphate assimilation) pairs. TCSs also control the transport of tricarboxylates and C4-dicarboxylates. In *P. aeruginosa*, out of 129 regulators (HKs and RRs), only a few of them have been studied in detail (Table 1). It is somehow difficult and risky to infer biological functions of proteins, only based on sequence similarity. Therefore, only recently discovered and well-identified systems will be discussed in this section.

4.1.1. Ethanol Metabolism

P. aeruginosa can grow on ethanol. The genes coding for the ethanol oxidizing system, form the *exaABC* gene cluster. Transcription is regulated by an HK, ExaD, which is probably located in the cytoplasm and a RR, ExaC[30]. The phenotypic characterization and transcription studies of several mutants indicate that seven different genes in a hierarchical organization may be involved in regulating the transcription of the ethanol system and components of acetate metabolism in *P. aeruginosa*.

4.1.2. Phosphate Starvation

The Pho regulon is regulated by the environmental Pi level. In *E. coli*, this control is coupled to an early step in Pi metabolism. The Pho regulon in *E. coli* has been extensively studied and constitutes now a paradigm[81]. The transcriptional activation of the regulon in response to Pi limitation requires two proteins, PhoB, the RR, and PhoR, the HK. Genes homologous to *phoB* and *phoR* have been characterized[5]. Several enzymes are synthesized upon Pi starvation, among which are an alkaline phosphatase and several phospholipases[54].

Two other signal transduction pathways also control the Pho regulon in *E. coli*[81]. Both of these controls are Pi independent. They are instead highly regulated by the carbon and energy source. One Pi-independent control requires the catabolic regulatory sensor CreC (a PhoR homolog formerly called PhoM). Like PhoR, CreC is autophosphorylated in an ATP-dependent manner and acts as a protein kinase of PhoB and its own partner RR CreB. The other Pi-independent control requires acetyl phosphate synthesis[81]. In absence of any detailed study, it is difficult to affirm that an analogous regulation mechanism exists in *P. aeruginosa*. Nevertheless, genes homologous to the *cre* operon are also present on the PAO1 genome, particularly CreC (PA0464).

4.1.3. Iron Starvation

Upon iron restriction, *P. aeruginosa* produces several specific devices in order to acquire the amount of iron necessary for growth. The ferric uptake regulator (Fur) plays a central role in this response and also controls other regulatory genes such as *pvdS*, which encodes a specific alternative sigma factor. Two TCS pairs also play an important role in iron acquisition. PfeR (RR) and PfeS (HK) are required for the enterobactin-inducible production of the ferric enterobactin receptor PfeA[20]. A second low-affinity ferri-enterobactin uptake

system exists in *P. aeruginosa* and may involve the PirA receptor together with the PirR–PirS regulatory systems[78].

4.1.4. Nitrogen Starvation

The large set of genes and operons regulated by the availability of nitrogen is known as the Ntr regulon, controlled by NtrB and NtrC in *E. coli* and other bacteria[51]. Two homologous genes have been identified in the *P. aeruginosa* genome (PA5124 and PA5125). Even though detailed studies are limited to date in *P. aeruginosa*, their role in regulation of nitrogen starvation is probably very important.

4.1.5. Carbon/Nitrogen Ratio

A novel TCS, CbrA–CbrB was recently discovered[53]. This system controls the expression of several catabolic pathways and, perhaps, together with the NtrB–NtrC system, appears to ensure the intracellular carbon nitrogen balance in *P. aeruginosa*. The signals that determine the activity of the CbrA–CbrB system are unknown. Understanding the signals and the target promoters of CbrB would provide deeper insights in the nutritional versatility and global catabolic regulation of *P. aeruginosa*. This TCS seems also to be involved, more or less directly, in the regulation of the operons for polyamine uptake and utilization in PAO1[42].

4.2. Signal Transduction in the Control of Operons Encoding Aerobic Respiratory Enzymes and Nitrate- and Nitrite-Responsive Gene Expression

P. aeruginosa is commonly classified as a strictly aerobic bacteria. However, oxygen limitation and the presence of nitrate are known to induce denitrification via the anaerobic respiratory pathway in this bacterium[19]. As in *E. coli*, the NarX/NarL system of *P. aeruginosa* could be involved in these energetic pathway. It was shown that, in contrast to *E. coli*, *P. aeruginosa* forms most of its heme under anaerobic denitryfying condition. The genes encoding the oxygen sensor Anr, the redox regulator Dnr, the nitrate regulator NarL and the DNA-bending IHF protein are all required for the cooperative anaerobic induction of the *hemA* gene, the first enzyme of heme biosynthesis[41]

4.3. TCS Signal Transduction and its Role in the Expression of Bacterial Virulence Factors

Pathogenic bacteria coordinate an intricate network of virulence factors, whose expression must be precisely controlled to maximize the chance of

establishing a successful infection. Adherence factors, toxins, capsules and other complex properties (e.g., motility, intracellular invasion) are among the virulence factors used by different pathogens. In addition, many pathogenic microbes display tactics for evading the host immune system. Not all virulence determinants confer a selective advantage to the bacteria at the same stage of infection, and so expression of these factors must be precisely timed. Groups of virulence factors acting at the same stage of the infection are often expressed in a coordinate fashion via common regulatory systems. Presumably, these regulatory systems recognize environmental cues during the transition from environmental reservoir to host and subsequently throughout the infection process. For example, temperature, pH, chemotactic signals, iron and so on.

Virulence is multifactorial in *P. aeruginosa*. A very large number of virulence factors have already been identified and *P. aeruginosa* shows a very broad host range. Indeed, it proliferates within hosts as varied as plants, insects, nematodes and mammals and interacts with amebae and yeasts as well. This broad host range has been used to uncover the role of new virulence factors and to characterize new regulatory systems controlling virulence.

Some TCSs systems, already discussed in Section 4.1 for sensing nutrients, are involved in regulation of bacterial virulence. The iron regulon controls expression of several virulence factors, including siderophores, exotoxin, proteases and hemolysin[78]. Besides, several factors, important for virulence, are under the control of the Pi regulon. Alginate synthesis, an important virulence factor in the case of CF, is under the control of several regulatory proteins, among which are two TCSs, AlgZ–AlgR and KinB–AlgB[22, 87]. Wang *et al.*[80] described a novel TCS that up-regulates the permeability of the membrane to various antibiotics, presumably by turning on the synthesis of membrane proteins that mediate antibiotic transport or by turning off efflux systems. The *pprA–pprB* regulatory system may represent a new mechanism for the control of membrane impermeability in *P. aeruginosa*. Moreover, it appears to be important during biofilm development[86].

Drenkar and Ausubel[24] identified a regulatory protein (PvrR) that controls the conversion between antibiotic-resistant and antibiotic-susceptible forms of bacteria. Compounds that affect PvrR function could have an important role in the treatment of CF infections.

Flagellar motility, type IV pili and twitching motility are important in pathogenesis. They are controlled by a range of regulatory systems including TCS (PilS–PilR, FleS–FleR) and a complex chemosensory system (Table 1)[27, 35, 62].

D'Argenio *et al.*[17], using the fruitfly *Drosophila melanogaster* as model host, have identified genes important for virulence, the *pilGHIJKL chpABCDE* gene cluster known to be required for twitching motility and potentially encoding a signal transduction system[46]. In their study, the *pil* genes and the *chp* genes appear to control the expression of additional

virulence factors, since twitching motility itself was not required for full virulence in the fly. ChpA is homologous to the central transducer protein and is similar to *Myxococcus xanthus* FrzE in that it contains a C-terminal CheY-type domain, but is unusual in that it contains six histidine phosphorylation[2].

In another study, D'Argenio *et al.*[16] demonstrated the activity of a novel RR, WspR, belonging to a chemosensory pathway, in autoaggregation of cells. This function is linked to expression of genes that encode a putative fimbrial adhesin required for biofilm formation[77]. WspR homologs regulate cell surface factors, including aggregative fimbriae and exopolysaccharides in diverse bacteria.

4.3.1. The PhoP/PhoQ System

The TCS PhoP–PhoQ has a major role in the virulence of *S. typhimurium* and regulates the expression of numerous virulence factors in this bacterium[32]. The periplasmic domain of PhoQ (HK) has been characterized as a divalent cation sensor (for a review see, ref. [31]). In *P. aeruginosa*, the PhoP–PhoQ homologs regulate expression of the outer-membrane protein OprH and polymyxin B resistance[45]. It also has a role in resistance to antimicrobial cationic peptides and aminoglycosides[44]. Nevertheless, it does not appear that PhoP and PhoQ have a role in virulence as crucial as the one they have in *S. typhimurium*.

4.3.2. The GacS/GacA System

GacS is important for swarming motility, lipase production, hydrogen cyanide biosynthesis and virulence in mice[15]. *P. aeruginosa* killing of *Caenorhabditis elegans* was used to identify virulence factors and GacA was identified as an important factor of virulence[74]. Later studies also showed it to be a multihost virulence factor and is also essential for biofilm formation[58]. From many studies, the Gac system seems to exert a very global control on virulence and GacS has evolved to have partial overlaps with PhoQ, a less-related virulence-associated HK[12].

5. STUDYING GLOBAL RESPONSE AND REGULATORY NETWORKS: MODULATION IN TCS GENE EXPRESSION

Adaptation by an organism to an environmental stimulus is not simply a group of independent responses by individual gene, but rather a coordinated series of linked events. TCSs mediate responses to a large variety of

environmental changes. To adapt and survive complex environmental changes in nature, it may be particularly important that different TCSs form regulatory networks and show dependencies and regulatory hierarchies. Until recently, little was known about functional interactions between different His–Asp phosphotransfer systems forming signaling networks.

Perhaps, the most difficult tasks in studying global regulation was determining the appropriate experiments to elucidate the components of the network, their interactions, and determining the temporal sequence of the responses. Recent technological advances have made it possible to study global gene expression by using high-density oligonucleotide microarrays. This microarrays technology opened up new perspectives for studying global regulations. This technology has already been used in a few cases and these studies offered a glimpse into gene regulation of TCSs.

5.1. Microarray Analysis of Global Gene Expression in Mucoid *P. aeruginosa*

Firoved and Deretic[28] applied microarrays analysis to identify at the whole genome scale those genes that are coinduced with the AlgU sigmulon upon conversion to mucoidy. Gene expression profiles of AlgU-dependent conversion to mucoidy revealed no obvious regulation of any TCS. Even the well-documented regulators of alginate production, AlgZ (FimS) and AlgR, did not show signs of an important differential expression between mucoid strain PAO5781 and its non-mucoid parental strain PAO381 (1.8–1.4 fold; Table 4).

5.2. TCS Gene Expression in *P. aeruginosa* Biofilms

Whiteley *et al.*[86] also used DNA microarrays to gain insights into differences between free-living cells and those in biofilms. In the experimental conditions they used, only about 1% of genes showed a differential expression between the two growth modes, despite their striking differences in lifestyles. Among the affected genes, only one TCS RR, *pprB* (PA4296), was repressed by a factor of 2.2 in the biofilm lifestyle (Table 4).

5.3. GeneChip Expression Analysis of the Iron Starvation Response

Iron starvation leads to a large response characterized by the derepression or induction of many genes involved in iron acquisition. This reflects well

Table 4. Genes whose expression was identified as being regulated in different growth conditions, by using microarrays.

Gene or ORF	Function	Name	Change	References
In biofilms				
PA 4296	RR	*pprB*	2.2	[86][a]
In mucoid cells				
PA5261	RR	*algR*	1.4	[28][b]
PA5262	HK	*algZ*	1.8	
Iron starvation				
PA0929	RR	*pirR*	6	[54][c]
PA0930	KK	*pirS*		
QS-regulated TCSs				
PA0179	RR		3.7	[66][d]
PA1976	HK		np[e]	[79][f]
PA1992	HK		np	[79][f]
PA2572	RR		3.3	[66][d]
			aerobic$^+$/anaerobic$^-$	[79][g]
PA2571	HK		aerobic$^+$/anaerobic$^-$	[79][f]
			2.1	[79]
PA3346	RR		4.7	[66][d]
PA4293	HK	*pprA*	6.6	[79]
			aerobic$^+$/anaerobic$^-$	[79][f]
PA4296	RR	*pprB*	3.4	[79][f]
			3.6	[66][d]
			5.6	[66][h]

[a]Strain PAO1 cultured in chemostat, in the presence or absence of granite pebbles to induce biofilms formation.
[b]Mucoid strain PAOR5781 (*mucA22*) and its non-mucoid WT parental strain, PAO3 (*mucA*$^+$).
[c]Increased expression upon iron limitation, low/high.
[d]Changes in gene expression in the signal generation mutant *lasI*$^-$ *rhlI*$^-$ grown in the presence of C^4HSL and $3OC^{12}$HSL compared with the absence of signal.
[e]Value not published.
[f]Changes in gene expression in the mutant Δ*lasI* Δ*rhlI* induced compared to uninduced growth conditions.
[g]QS-regulated transcripts detected at the early stationary phase in PAO1 grown under various nutrient and oxygen conditions.
[h]Changes in gene expression in the WT strain compared with the receptor mutant Δ*lasR* Δ*rhlR*.

the importance of this nutrient for growth and pathogenesis. Only one pair of TCS (*pir*S and *pir*R) appeared to be regulated positively (by a factor of 6) in conditions of iron starvation[54]. The expression of the other identified pair of TCS involved in iron regulation (namely *pfe*S and *pfe*R), was not affected by iron starvation. It is important to note that the observed change for *pirS* and *pirR* is relatively large (6 fold) compared to those observed in biofilm and mucoid cells (2.2 and 1.6 fold) respectively (Table 4).

5.4. Quorum Sensing Control on TCSs Expression

Regulation by quorum sensing (QS) is very important in *P. aeruginosa*. QS has been shown to regulate the production of virulence factors and is involved in biofilm formation and development (for a review, see ref. [21]).

Microarrays analysis of *P. aeruginosa* QS regulons were recently performed in two laboratories[66, 79]. The experimental conditions used were different and gave different insights in QS regulation. Besides identification of regulated genes, the effects of growth phase and environment, and the signal specificity were also observed. Between 6% and 10% of the total genome appeared to be regulated, depending on the strains used and on the experimental conditions of growth. This large percentage of genes identified in both studies as being QS regulated reflects the global level at which QS influences cellular behavior.

A certain number of TCS genes were identified (Table 4), most of them without any known function. Only a few of them were identified in both studies, which emphasizes further the variation in expression of some TCS genes dependent upon culture conditions, environmental conditions and timing of their expression.

5.5. Regulatory Networks and Cross Regulation

A total of eight TCSs were shown to be QS regulated which represents 6% of the total TCS (four HKs and four RRs; Table 4). One pair of regulators was found regulated by QS: *pprA* (PA4293) and *pprB* (PA4296), and the same RR, *pprB*, was also identified as regulated in biofilms. Moreover, the expression of the four HKs was modulated when PAO1 was cultivated aerobically and anaerobically in various media[79].

From those studies, it is clear that expression of some TCS genes is variable depending on other global regulators. Probably, more examples of TCS regulation will come up soon with an increasing number of studies. At this point, it is nevertheless interesting to note that the observed cross regulations are variable. Mucoidy, development as biofilm and iron starvation affect only a minimal number of TCS genes, very specific of the type of regulation studied. In contrast, QS regulation has the largest impact on TCS expression, on both HKs and RRs. Keeping in mind that one TCS (GacS/GacA) can also modulate QS regulation[61], these studies strengthen the concept of regulatory networks.

Cross regulation refers to the control of an RR of one TCS pair by a different regulatory system. By definition, cross regulation controls the activity of the RR. Also, it is likely that cross regulation always involves the control of an RR by phosphorylation (or dephosphorylation). However, cross regulation may

involve different mechanisms[81]. Before studies with microarrays, one example of cross regulation was well characterized. Two of the major virulence determinants are type-4 pili and the exopolysaccharide alginate which cause the cells to become mucoid in CF patients[84]. It was demonstrated that the major RR of alginate synthesis, AlgR, is also involved in fimbrial biogenesis and twitching motility[84]. Immediately upstream of *algR*, lies the gene encoding its cognate sensor, *fimS*, which is also required for fimbrial biogenesis, but, surprisingly not for alginate production. AlgR and FimS constitute a new class of HK/RR pair, along with YehTU from *E. coli* and LytSR from *S. aureus*. An additional level of cross regulation was demonstrated when a *pilS* mutant was also found to be defective in alginate production[2]. It appeared clearly from this example that *P. aeruginosa* has the ability to sense the appropriate host environment and coordinately regulate two of its major virulence factors. This network may possibly extend more broadly to the control and integration of the expression and function of other factors involved in host colonization.

6. CONCLUSION

In this chapter, we have summarized our current knowledge about TCSs to highlight the questions that should be addressed in future research on *P. aeruginosa* biology and pathogenicity. From previous studies[63, 68], two striking features already emerged. The first was the high number of TCSs retrieved in the *P. aeruginosa* genome. The second striking feature was the presence of many atypical kinases involved in multistep His–Asp phosphotransfers that allow multiple regulatory checkpoints, providing a high level of control during signal transduction. The presence of complex control strategies in response to different environmental challenges is likely to be crucial for this most versatile bacterium.

Although the contributions of many investigators and laboratories enable a first view on the function, interaction and regulatory potentials of a limited number of TCSs, we are just beginning to understand the regulatory networks in *P. aeruginosa*. Nevertheless, two important features have emerged:

- Some TCS genes identified as regulated are themselves regulators, therefore forming a *regulatory cascade*. It will be important to investigate their position in the hierarchy of the cascade.
- There are evidence that functional interactions exist between different TCSs, such as *cross regulation*, allowing cooperative functioning between TCSs. Whether these interactions are direct or indirect remains to be investigated.

Because TCSs form networks that involve more than one system and show dependencies and hierarchies, they are likely to have important roles in *P. aeruginosa* cell physiology and to be adapted for very complex processes. The major themes that emerge from recent studies on TCSs are those of complexity and diversity of regulatory networks.

As already performed in a limited number of studies, additional studies will have to be done. First, we will have to identify complete regulons from each of RR or TCS, hoping that the probable function of some unknown TCS could be deduced from the list of their target genes. Second, the characterization of TCS sensor specificity and input signals will be very helpful. Third, the elucidation of the complex phosphorelays will have to be done. And, lastly the cross regulation including regulatory hierarchies, overlaps and superimposed regulators have to be elucidated.

These studies enable us to begin the elucidation of signal transduction networks potentially active in *P. aeruginosa*. This should aid in a more detailed understanding of its virulence in the near future and perhaps, a more accurate prediction of disease outcomes. Identifying regulatory networks is essential if the goal is to predict or control a cell's behavior.

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10

TRANSCRIPTION MEDIATED BY *rpoN*-DEPENDENT PROMOTERS

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1. INTRODUCTION

Prokaryotic promoters dependent on the alternative sigma factor σ^{54} are a kind on their own. They employ a distinct mechanism for transcriptional activation which is altogether different from any of the others transcribed by the enzyme bearing the housekeeping sigma factor (σ^{70} in most Gram-negative bacteria) or any other type of sigmas. As explained in more detail below, the outstanding features of σ^{54} promoters include: (a) their activation at a distance by regulatory proteins bound relatively far upstream from the binding site of the σ^{54}-containing RNA polymerase (RNAP), (b) the formation of a non-productive closed complex of the enzyme with the target DNA in the absence of activation, (c) the looping out of the intervening DNA region placed between the upstream activator (protein binding) sequences (UAS) and the downstream site for the σ^{54}-containing RNAP, (a) the need of activator oligomerization and ATP hydrolysis for initiation of transcription and (e) the ability of the σ^{54}-dependent regulators (at least some and under some conditions) to activate transcription from solution—that is, in the absence of any apparent binding to DNA.

Although initially believed to be associated only to N-metabolism, it is now clear that σ^{54}-promoters regulate a wide variety of unrelated functions.

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Why some systems depend on σ^{54} instead of other regulatory assets is still a matter of discussion. Yet, as argued below, such promoters can process a large variety of environmental signals by a combination of protein–protein interactions and protein-induced changes in promoter DNA geometry.

This chapter will address the general architecture and functioning of σ^{54} promoters, as discrete entities and as a whole. Although the focus of this article is *Pseudomonas*, we shall illustrate key features of these promoters in the experimental systems (frequently other Gram-negatives) where the point has been best substantiated. Along the line, we will review the functions known in various *Pseudomonas* species to be regulated by a σ^{54}-dependent control mechanism. In many cases, we will refer to the σ^{54} promoter *Pu* of the catabolic TOL (toluene/*m*-xylene) biodegradation pathway of plasmid pWW0 of *Pseudomonas putida* mt-2. *Pu* was the first *Pseudomonas* promoter identified as being dependent on *ntrA*[25], an earlier designation of gene *rpoN*, encoding σ^{54}. Over time, the *Pu* promoter, along with the similar *Po* promoter of the methyl-phenol degrading strain *Pseudomonas* sp. CF600 (see Chapter 16 of this book) has evolved as the experimental system of choice to address many general issues of σ^{54} systems. Finally, we will discuss the evolutionary value of this type of promoters as instruments for processing and molecular signalling of environmental inputs.

2. THE SIGMA FACTOR σ^{54}

The discovery and functional assignment of the σ^{54} factor and its dependent promoters have an enticing narrative. The gene encoding what later turned out to be an alternative sigma factor of RNAP was first discovered as one chromosomal site involved in N_2 fixation in *Klebsiella*[69] and general N metabolism in *Esherichia coli*, and *Salmonella*[88]. The same gene was renamed in various laboratories but the specific role remained elusive for some time. Two separate sets of observations in *E. coli* eventually converged into the same place. On the one hand, Magasanik's group reported in 1986[73, 90] that the promoter driving the gene for glutamine synthetase of *E. coli* could be transcriptionally activated at a distance from a sequence in a fashion not unlike that known for eukaryotic counterparts. Such a promoter was dependent on two factors, the lack of one of which abolished expression of all N-uptake related genes and a second which was needed only for expression of the glutamine synthetase gene. At that time, these two papers[73, 90] challenged the generally accepted view that transcriptional regulation occurred from sites proximal to the RNAP binding sequence. At a later moment, the gene was sequenced, the protein purified and an in vitro assay set up which clearly demonstrated that NtrA encoded a sigma factor[88]. This was designated alternatively as σ^{N} (as it was

believed that it controlled the N regulon) or σ^{54} in reference to its apparent molecular size (although it migrates abnormally in a denaturing polyacrylamide gel). According to genomic information available in the databanks σ^{54} is present in most, but not all, Proteobacteria[100, 103], *Haemophilus influenzae* being a notable exception. However, it is not found in genome sequences of any of the cyanobacteria, *Deinococcus* group, *Thermotoga*, and most of the Gram-positive bacteria (streptococci, staphylococci, mycobacteria, mycoplasmas and lactic acid bacteria). Yet, it is present in *Bacillus subtilis*, where the σ^{54} homologue has been called SigL[23]. Some bacteria have two copies of the gene, although in general, only one is to be found. Subsequent work made predominantly with a variety of promoters of *E. coli* and N-fixing *Klebsiella pneumoniae* strains established the basic features of σ^{54}-dependent systems which, with little variations, are common to all of them[11, 74].

At least in the case of *P. putida*[20, 60], with similar counterparts in other *Pseudomonas* species[49], the *rpoN* gene is located in the genome adjacent and perhaps co-transcribed with four open reading frames (ORFs), two of which (named *ptsN* and *ptsO* genes) encode proteins similar to components of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The products of these genes are believed to modulate the activity of some σ^{54}-dependent promoters in response to the presence of distinct C sources in the medium (see Chapters 13 and 18 of this book). In *Pseudomonas aeruginosa* the loss of ORF154 (*ptsN*) had no effect on the expression of pilin or flagellin genes (which are dependent on *rpoN*, see below), but cells grow much worse in minimal medium[49]. Since the latter effect was suppressed by addition of glutamine (but not other amino acids), it was proposed that such a gene might act as a coinducer of some σ^{54}-controlled genes[49]. This issue is not yet clear and deserves future attention. A clearer feature of *rpoN* expression is that, at least in the case of *P. putida*, the *rpoN* promoter seems to be autoregulated: *rpoN* promoter expression is five times higher in the *rpoN* mutant[60]. This may cause the intracellular levels of the sigma factor to remain invariable through all types of growth conditions[54]. The σ^{54} protein is synthesized in a form able to unite the core RNAP and bind stably promoter DNA as part of the holoenzyme. This binding, however, does not result in activation unless the sigma factor is contacted and turned on by the cognate regulator bound upstream (see below).

Despite the lack of a tridimensional x-ray structure of σ^{54}, sequence alignments, mutation analyses, and protein fragmentation studies have led to a picture of the overall domain structure of the protein (see ref. [11]). The factor can be divided into three regions. The N-terminal ~50 residues (region I) comprise a domain that inhibits polymerase isomerization/initiation in the absence of activation and stimulates initiation in response to activation. In many cases, this is followed by the acidic region II which has been implicated

in DNA melting in the transition from the closed to open complex and in assisting σ^{54} binding to homoduplex and heteroduplex DNA. Yet, this region II may not be acidic (e.g., in the σ^{54} homologue of *B. subtilis*, SigL) or may even be absent (e.g., *Helicobacter pylori* σ^{54}). Finally, region III contains the core RNAP binding determinants. Further, region III encompasses the primary DNA-binding functions, with a DNA cross-linking segment and associated motifs: the helix–turn–helix motif and the *rpoN* box near the C terminus. Adjacent sequences modulate the activities of these regions[11].

3. ORGANIZATION AND FUNCTIONING OF σ^{54}-DEPENDENT PROMOTERS

Promoters dependent on σ^{54} are characterized by a number of distinct elements that include DNA sequence motifs and cognate regulatory proteins as well as nucleoid-associated factors. In a limited number of cases, these elements have been identified through traditional wet procedures. But the advent of genomics allows increasingly the access to the σ^{54} sigmulon of many bacteria on the only basis of computational analysis of their DNA sequences and their predicted proteins[26, 89, 99–101, 103].

3.1. The DNA Sequence Motifs in σ^{54} -Dependent Promoters

Promoters of this kind typically span DNA segments of about 150–200 bp. The most outstanding feature of σ^{54}-systems is the presence of sequences characteristic for binding of the σ^{54}-containing RNAP, the so called $-12/-24$ motifs (Figure 1). These include GG and GC doublets at positions -24 and -12 respectively instead of the typical -10 and -35 hexamers of the σ^{54} -promoters.

The $-12/-24$ motif acts as the docking site for the enzyme, with which they can form non-productive closed complexes. The specificity of the σ^{54}-RNAP for the $-12/-24$ sequences is determined by the sigma factor itself and, at least in some promoters, even the isolated sigma can form a complex with the corresponding sequence in the absence of the core enzyme[10, 13]. In other cases, formation of the closed complex requires the assistance of auxiliary proteins for recruiting the polymerase to the promoter[7, 15, 67].

The second major functional motif in σ^{54}-promoters is the binding site(s) for the cognate activators. These sites may be located at various distances (50–150 bp) upstream of the $-12/-24$ motif. Again, this is different from regulators of promoters transcribed by RNAP forms bearing other

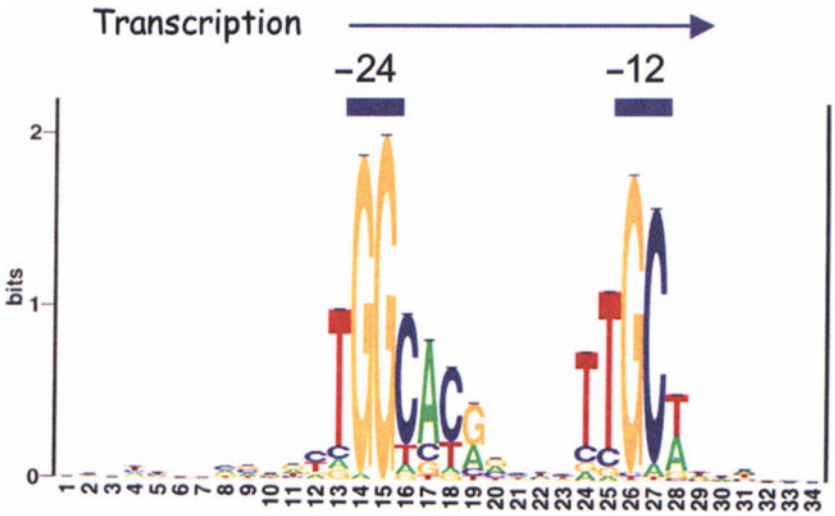

Figure 1. The DNA sequence motif recognized by the σ^{54}-containing form of RNAP. The figure summarizes the most frequent bases found at and around the −12/−24 sequences of *rpoN*-dependent promoters.

sigmas, the sites for which used to be adjacent to the binding of the enzyme. On the contrary, the regulator binding sites of these promoters are reminiscent of the UAS (enhancers) of eukaryotic promoters. On this basis, regulator binding sites in σ^{54}-promoters are generally designated as the (prokaryotic) enhancer region or the UASs (Figure 2). Since only a few sites have been studied in detail, it is difficult to come up with a general sequence signature for the UAS[21].

Yet, the way the cognate proteins bind them (see below) generally involve at least two phased palindromes (~16 bp each) separated by various distances (Figure 3). Genomic identification of such sequences is, however, hampered by the potential existence of cryptic sites which may be occupied at higher concentrations of the regulator and also by the fact that some promoters may be activated by their cognate activators from solution, that is, in the absence of any evident DNA binding[70, 115]. The operative function of the UAS is thus to increase the local concentration of the regulator in the proximity of the DNA-bound σ^{54} -RNAP.

In addition to the −12/−24 and the UAS, many (but not all) promoters have an integration host factor (IHF)-binding site at the intervening region (Figure 2). IHF binding sites include the motif 5′WATCARNNNNTTR3′ (where W is A or T and R is A or G) separated by 8 bp from a less conserved A/T-rich track of 4–6 bp. IHF binding to DNA sharply bends the target sequence, an event that has the multiple consequences discussed below.

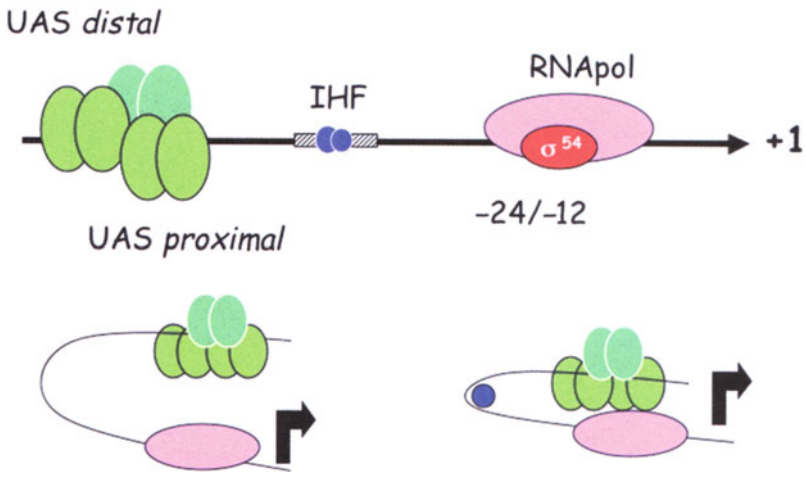

Figure 2. General organization of *rpoN*-dependent promoters. The distribution of relevant DNA sequences is shown. These include the UAS for the enhancer-binding proteins (EBPs) and the −12/−24 region recognized by σ^{54}-RNAP. Some promoters contain a functional integration host factor (IHF)-binding site located within the intervening region. Protein sizes are symbolic. The upstream nucleoprotein complex may contain 4–8 monomers of the regulator (most likely, it is a hexamer[120] or a heptamer[65b]).

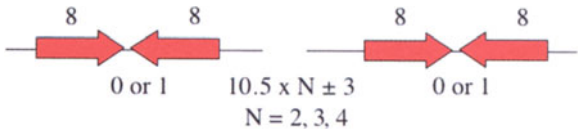

Figure 3. Numerical description of the UASs which act as binding sites for the EBPs. The general organization of the UAS seems to follow the regularity indicated[21].

The first reports on the presence of IHF sites in σ^{54}-promoters[1, 22, 42] suggested that the one role of IHF binding was to fix an optimal promoter geometry facilitating contacts between distant proteins. Yet, subsequent research has revealed a repertoire of regulatory and physiological effects brought about by IHF on promoters of this kind.

Regardless of the presence of IHF, the set-up of σ^{54}-promoters is such that the upstream nucleoprotein complex formed by the UAS and the EBPs must contact the σ^{54}-RNAP bound downstream in −12/−24 (Figure 2). This implies that the intervening sequence has to bend one way or the other for generating the required promoter architecture. The intervening DNA must thus be *bendable*, a property that is not granted to every sequence[85]. DNA does not behave as an isotropic rod: depending on the sequence, it may bend more easily in one plane than another and thus possesses a degree of anisotropic

flexibility. One important difference between curved DNA and *bendable* DNA arises from their different physical dynamism. Statically curved DNA is already deformed even in the absence of external forces, thereby resulting in a rigid structure. On the contrary, *bendable* DNA allows a mixture conformational states, the equilibrium of which can be displaced towards one specific form by external forces such as proteins interacting with them. Many regulatory proteins (mostly nucleoid-associated factors) tend to bind pre-curved or bending-prone sequences[85]. This is the case of HU, which binds and helps to bend the intervening region of some σ^{54} -promoters in the absence of a defined target sequence[82, 84].

The summary of functional DNA segments of σ^{54}-promoters is shown in Figure 2. While $-12/-24$ signatures and $-12/-24$ sites are simple to detect in bacterial genomes[103], their combination with UAS and pre-curved or *bendable* DNA sequences requires elaborated bioinformatic and computational approaches[21]. The number of σ^{54}-systems may further increase if we consider that some promoters have their UAS downstream of the RNAP binding site[55]. Others may have UAS much further upstream[47]. There might be also perfectly concomitant σ^{E} and σ^{54} sequences[8], a combination of σ^{70} and σ^{54} (see ref. [57]) or an overlap of divergent σ^{70} and σ^{54} (see ref. [68]).

3.2. Transcriptional Activator Proteins Acting in Combination with σ^{54}

Apart of the sigma factor itself, the main players of σ^{54}-dependent systems are the cognate regulatory proteins which bind the UAS and ultimately activate transcription. Given the reminiscence to eukaryotic systems, these UAS-binding activators are frequently designated as *prokaryotic enhancer-binding proteins* or EBPs. All of such activators share a good deal of similarity in their structure and mode of functioning. The regulators belonging to this group, generically known as well as the *NtrC family* of proteins, are composed of four domains, the characteristics of which are disclosed separately below. As shown in Figure 4, the domain structure generally follows the sequence A (signal response)-B (linker)-C (activation)-D (DNA binding). This reflects the modular organization of this type of proteins, a feature that is very unusual in prokaryotic regulators—but frequent in eukaryotic transcriptional factors. Exceptionally[8b], some EBPs can activate σ^{70} promoters in a fashion not dependent on ATP hydrolysis, but such cases will not be addressed here.

3.2.1. The Signal Response A Domain

In most (but not all) cases, the amino-terminal module (A domain) is the signal response domain and is used to be the most variable among the regulators, since it is the one that receives directly or indirectly the input leading to

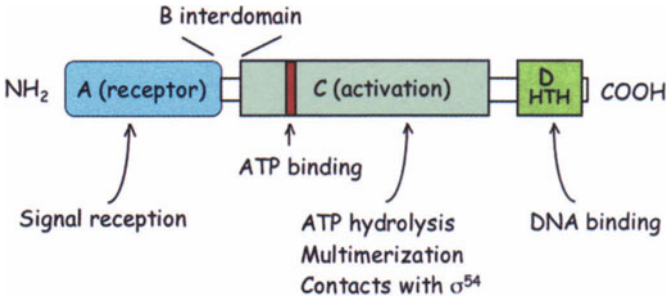

Figure 4. Domain organization of the EBPs which act in concert with σ^{54}. Relevant portions of the proteins include the signal reception, N-terminal A domain, the central (C) module involved in NTP binding, and the D domain at the C-terminus, with a helix–turn–helix (HTH) motif for DNA binding.

the activation of the protein into a transcriptionally competent form[95]. The sizes of such A domains vary from few amino acids to a large structured protein module. According to wet experiments and *in silico* sequence analysis[102], major classes of A modules include: (a) PAS signatures (involved in signal transduction communicating ligand binding/activation to downstream transducer proteins), (b) response domains (substrates of phosphorylation by sensor proteins in two-component systems), (c) GAF modules (cyclic nucleotide recognition folds), (d) ACT domains, for example, regulatory modules frequently linked to metabolic enzymes regulated by amino acid concentration[2] and (e) V4R, which typically bind aromatic chemicals[95]. PAS and ACT folds may lie adjacent within the same A domain of certain proteins[21].

GAF, PAS and V4R domains bind directly small molecules as effectors, although the consequences of such a binding may vary in each case[102]. EBPs bearing these domains generally activate transcription without any other protein intermediate. Those bearing a V4R-type A domain become constitutively active when the A domain is removed, thereby suggesting that activation brought about by aromatic effector binding is the result of relieving an intraprotein repression caused by the A domain on otherwise activating protein surfaces[95]. The structure of some V4R domains (Figure 5) has received much attention in recent years in view of the possibilities to reshape their effector binding pocket(s) through directed protein design and combinatorial approaches[24, 34]. This may allow the selection of protein variants capable of responding to predetermined chemicals (regulators *á la carte*).

Other EBPs bear A domains which are phosphorylated by second sensor protein. In these cases, phosphorylation is required for activity, although the downstream consequences of such an event may vary among EBPs. For instance, while removal of the phospho-acceptor A domain of NtrC inactivates the protein altogether[64], deletion of the same phosphorylable module of DctD

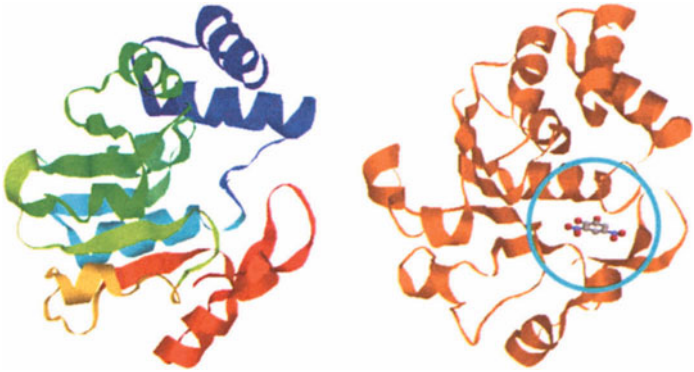

Figure 5. Structural model of the V4R-type A domain of the XylR/DmpR-type EBPs. A 3-D model was built for the A domain of XylR and XylR-like proteins by homology with a family of mammalian COMT[24]. The model passed standard tests of normality for protein structures, but it is more reliable in the regions in and adjacent to the predicted binding pocket for the aromatic effectors, which are common to all members of the XylR/DmpR family. Note the position of the aromatic effector in the predicted structure[24].

originates a constitutive protein[65]. Other A domains (characteristically those of NifA-like proteins) modulate their activities through protein–protein contacts. Finally, there are some EBPs which appear to lack any significant A domain[74, 102]. In these cases, the protein may be born constitutively active or may be down-regulated by yet another factor interacting with the other protein domains.

3.2.2. The Central Domain C

This is the most conserved region among the protein family members, since it is involved in the binding and hydrolysis of ATP that is at the basis of the activation of the σ^{54} -promoters[114]. The sequence of the C domain makes this module to belong to the extensive AAA+ super-family of ATPases, members of which are found in all three kingdoms of life and function in diverse cellular processes, often via chaperone-like activities. The role of such domains in activation of σ^{54} has been reviewed in detail by Zhang *et al.*[120]. It seems that formation and collapse of the transition state during ATP hydrolysis is the key event which triggers the interaction of the activator proteins with σ^{54} and leads to the structural transitions in the sigma factor which are needed for RNAP to isomerize and engage productively with the DNA. AAA+ proteins possess an oligomeric (e.g., hexameric or heptameric) structure, the active site for ATP hydrolysis being located between monomers[65b,120]. The changes in the oligomeric structure required for productive interactions with the σ^{54} holoenzyme appear to be a consequence of sensing the state of the γ-phosphate of ATP. Depending upon the form of nucleotide bound, different functional states

of the activator are created. These can maintain σ^{54} in various conformational states, so the mechanism by which regulators of this type activate transcription involve chaperone-like events[120]. From these observations it follows that the A modules generally fused to the C-domains of prokaryotic EBPs may exert their regulatory function by preventing ATP hydrolysis and/or the oligomerization of the AAA+ modules and/or the contacts of the oligomer with the σ^{54} holoenzyme[65b].

3.2.3. The Linker B Domain

A shorter connector (named the B linker) with different degrees of structure connects the C domain to the A domain. The role of this *hinge* connecting the two domains was initially considered as that of a mere linker to assist the contacts between the other two modules, with no direct contribution to the activation process. This notion was largely based on the early proposal that the abundance of glutamines (Q) through the homologous B regions of a number of EBPs could give rise to a flexible and non-structured coiled polypeptide (the so-called Q linker) with little chances of being more than an interdomain hinge. The lack of a detectable phenotype in various insertion mutants within such Q linker of NtrC and NifA substantiated this notion. However, more recent data with the XylR[33] and DmpR proteins[75] have shown that the interdomain B sequence is in fact actively engaged in fixing the protein in a form that is competent for transcription activation and in setting indirectly the structural tolerance of the protein for specific effectors. However, this may not be a general rule, as many other EBPs bear B domains which lack any significant structure.

3.2.4. The DNA-Binding Domain D

The C-terminal portion of most EBPs consist of separate module with DNA-binding abilities. This feature was first identified by just inspecting the presence of a helix–turn–helix motif in the corresponding polypeptides as deduced from their gene sequence. The specific protein folds of the C-terminal domains may vary, and at least in the case of NtrC, the DNA-binding module is similar to that of the nucleoid-associated Fis protein of *E. coli*[74, 102]. In NtrC, this region contains major dimerization determinants for the protein, while those of most other EBPs appear to reside in the AAA+ domain.

One intriguing aspect of the C-domains of the class of EBPs that respond to aromatic effectors is the conservation of the recognition helix of the HTH motif. This implies that the target DNA sequences are the same and therefore that such activators can cross-activate their cognate promoters—a feature that has been proven experimentally in a number of cases[30, 70]. In the other extreme, the DNA-binding domains of the proteins and the cognate UAS of some EBPs (typically the NifA-like proteins of N-fixing bacteria) are less defined. In these cases, the activation effect could occur from solution. Moreover, examination of the genomes of Pseudomonads reveal the existence

of proteins (for instance, GeneBank PP5116) which bear exclusively the central domain of EBPs, lacking both the A and D domains[21]. Whether these are involved in transcriptional activation from solution or in entirely different cell functions deserves further studies.

3.3. DNA Bending in σ^{54} Promoters

σ^{54}-promoters, which are activated at a distance (100–200 bp) by specific regulators bound to UAS that must loop out to contact the RNAP, make the intervening DNA sequence an ideal target for factors that promote or inhibit the correct promoter geometry required for activation. As mentioned above, many σ^{54}-promoters have integration host factor (IHF) sites at such intervening region. However, neither the precise location of the binding site, nor the influence of IHF is identical in all cases. IHF is a small (~20 kDa) basic heterodimeric protein that contains a helix–turn–helix domain involved in dimerization and two antiparallel β-sheets. IHF binds and sharply bends DNA, producing a distinct distortion on target DNA sequences. IHF sites are typically present at the intervening region between the UAS and −12/−24, but the precise location does vary and does have different effects. In the most typical cases the IHF is placed such that its assists the formation of a DNA loop between the UAS-bound regulators and the polymerase. This notion is supported by the possibility to effect functional substitutions of the IHF sites by intrinsically curved in the σ^{54}-dependent promoter *Pu* of *P. putida*[86] and the *PnifH* promoter of *K. pneumoniae*[85]. The lack of IHF abolishes altogether *Pu* activity in *P. putida*[12, 109] but not in *E. coli*[1] a fact that has been attributed to the fortuitous replacement of IHF by HU[84]. This architectural role of IHF is probable when the site is *strategically* positioned between the UAS and −12/−24. But reality is that IHF sites may be displaced towards one extreme of the loop and thus unlikely to act merely as a helper to structure the promoter sequence. IHF can perform as different functions as mediating the recruitment of the polymerase[7, 15, 67], or just the opposite: inhibiting binding of σ^{54}-RNAP[35, 68]. IHF can also increase[53] or impede[68] the binding of the EBPs to the UAS, and also stimulate formation of an open promoter complex[104], this last feature perhaps through a structural transmission mechanism. An intriguing function of IHF in some systems is to increase the fidelity of the promoter for a given regulator, thereby suppressing promiscuous activation caused from solution by other proteins of the same activator family[27, 81].

3.4. σ^{54} Promoters in Action

Once all the elements needed for activity of σ^{54} promoters are in place, how do they work? This question has an in vitro, mechanistic aspect, as well

as an in vivo angle. The standard view of these promoters depicts the corresponding DNA sequence engaged by the σ^{54}-RNAP at $-12/-24$, the UAS bound by the EBP and in some cases, the IHF factor bound in between (Figure 2). These factors may be stably docked in the DNA and still the promoter will be inactive until a given environmental signal acts on the A domain of the EBP, thus initiating a whole cascade of events which end in strand separation at the downstream sequence and transcription initiation[11, 120]. Activation of σ^{54}-RNAP occurs by signal transduction pathways initiated differently in each activator. Although these pathways are many, they converge in common terminal mechanism which involves the engagement of an ATPase activity within the EBP. Such ATPase is stimulated by the reception of the environmental signal by the A domain[96] and sometimes by DNA binding as well[83]. In other instances, though, the EBP may have a considerable intrinsic ATPase activity, and the reception of the signal by the A domain mostly causes the coupling of such ATP hydrolysis to transcription activation[4, 75].

Changing physiological conditions typically leads to drastic conformational changes in the protein's N terminus. These changes then can lead to an increased activator affinity for enhancer DNA sites, multimerization on the DNA, and most important, the assembly of a DNA-bound complex with ATPase activity. Although the details may vary for each specific system, the process generally implies unlocking of the activation potential of the central domain. This is brought about by major conformational changes in the A domain and the binding of ATP to the C module. This causes a concomitant oligomerization (most likely hexamerization[120] or heptamerization[65b]) of the activator. As mentioned above, ATP is then hydrolysed. The state of the γ-phosphate of ATP during hydrolysis is the key event which makes the EBP change the DNA-bound conformation of σ^{54} onto a protein species able to separate the DNA strands and initiate transcription[11, 120]. While the activity of NtrC and proteins alike are controlled by phosphorelay, many other activators, instead react to small-molecule effectors or inhibitory polypeptides. Readers are directed to the recent work of the Laboratories of S. Kustu[37, 64], M. Buck[120], B. T. Nixon[77, 78] and D. E. Wemmer[37, 56, 65b, 119] for details of this intricate process. Once transcription is initiated, the whole nucleoprotein complex may disassemble prior to starting another round[83]. This picture comes exclusively from experiments in vitro, in most cases made with DNA templates lacking the UAS and with purified, preactivated protein, but how does it translate to scenarios in vivo?

The main difference between in vitro and in vivo is that the binding sites for each of the major proteins are not necessarily occupied always by the cognate protein(s). The σ^{54} factor itself is a quite scarce protein, for example, ~80 molecules per cell in *P. putida*[54], which has to compete with other sigmas for accessing the intracellular pool of core RNAP. The apoenzyme is in limited

supply during stationary phase, and the populations of each of the holoenzyme forms is determined by ppGpp levels[52, 62]. The consequence of this is that only −12/−24 sequences with a high affinity for σ^{54}-RNAP may spontaneously form a stable closed complex when the physiological conditions do not result in increased ppGpp levels. Similarly, the number of EBPs in vivo may vary. A direct count of XylR monomers in *P. putida* yields as little as ~80 specimens per cell in exponential growth and ~140 in stationary[31]. These figures allow just a few active multimers to be formed. Direct measure of the occupation of the UAS by XylR with high-intensity UV imprinting (Valls and de Lorenzo[109b]) does reveal that the UAS are not significantly occupied at any time. But the most striking condition is that of IHF. The changes in intracellular concentrations of IHF in *P. putida* between exponential and stationary phase are quite pronounced[109]: from ~2000 to ~14000. This shows that promoters such as *Pu*, which is entirely dependent on IHF, are unable to show any significant activity until cells reach the stationary phase of growth. The IHF site of this promoter is not occupied until cells reach such a stage[109], thus causing the loss of an optimal geometry of the region and the inability to recruit the σ^{54}-RNAP to −12/−24 sequence. At least in *Pu* the consequence of all this is the coupling of transcriptional capacity to growth phase[109]. But in general, it is plausible that the binding of every protein to the corresponding σ^{54} promoter is subject to some type of control in vivo. This enters additional checks in the outcome of transcription under various physiological conditions. Some of such mechanisms have been examined in Chapters 13 and 18 of this book and will not be addressed here. But the consequence is that σ^{54} promoters may remain silent in vivo until cells reach an optimal physiological state which allows all molecular players to bind their partners and to adapt an optimal geometry.

4. σ^{54}-DEPENDENT PROMOTERS IN *PSEUDOMONAS*

The number and functions of σ^{54} systems vary tremendously among the various *Pseudomonas* strains where the issue has been addressed. Most studies have been made in *P. aeruginosa* as an archetype of human pathogen, *P. putida* as a typical soil species and *P. syringae/P. fluorescens* as cases of bacteria interacting negatively or positively with plants. In the first two cases, some new insights on σ^{54} promoters can be deduced from the analysis of their complete genomic sequences[100, 101, 103]. In the section below, a non-exhaustive catalogue of σ^{54}-dependent systems of various *Pseudomonas* species is discussed according to their major functions—instead of the specific hosts.

4.1. Role of σ^{54} in Nitrogen Metabolism

As mentioned above, nitrogen metabolism was the first function assigned to σ^{54}, to the point that the product of *rpoN* is frequently designated as σ^{N} or the N sigma factor. Indeed, *rpoN* mutants of *P. aeruginosa*[107] and *P. putida*[61] lack glutamine synthetase and fail to produce urease. But σ^{54} may be directly or indirectly involved as well in denitrification. For instance, an *rpoN* mutant of a *Ralstonia* strain (a honorary *Pseudomonas*) was defective in denitrification[123]. However, the equivalent mutation does not affect the same process in many other bacteria[36]. The *rpoN* mutant of *P. aeruginosa* can grow anaerobically in the presence of nitrate as electron acceptor. This rules out that *rpoN* is necessary for anaerobic utilization of nitrate in this species[107]. The situation is slightly different in *Pseudomonas stutzeri*. In this case, while *rpoN* is not involved in transcription of the reductase genes which form the denitrification system, reality is that the lack of σ^{54} decreased the cellular concentrations of nitrite reductase and NO reductase. It is plausible that the enzyme levels are subject to a post-translational control mechanism involving one or more factors, the expression of which depend on σ^{54} gene expression[36].

Besides acquisition of N from inorganic sources, σ^{54} participates also in various cases of utilization of amino acids in *Pseudomonas*. The proline dehydrogenase gene (*putA*) of *P. putida* negatively autoregulates its expression and that of the permease (*putP*) in the absence of proline, as seen in Enterobacteriaceae. But neither *putA* nor *putP* is expressed in an *rpoN* mutant, although the corresponding regulator is still unknown[111]. This regulation may be different from that observed in *P. aeruginosa*, in which *putA* and *putP* are co-expressed by a promoter regulated by PruR, a protein of the AraC/XylS family[71]. Following with proline metabolism, the *proC* of *P. aeruginosa* (encoding the third enzyme required for proline biosynthesis) and the *anr* gene (required for anaerobic regulation of nitrate respiration and arginine degradation) contain conserved $-12/-24$ boxes but appear to be independent of *rpoN*[93]. This illustrates the difficulty to assign genes to the σ^{54} sigmulon on the only basis of sequence signatures. The situation is clearer in the *phhABC* operon of *P. aeruginosa*, encoding the enzymes phenylalanine hydroxylase, carbinolamine dehydratase and aromatic aminotransferase, which is regulated by the σ^{54}-dependent regulator PhhR in concert with *rpoN*[98]. Finally, the lack of σ^{54} inhibits expression of the *ansB* gene of *P. fluorescens*, which encodes a periplasmic glutaminase/asparaginase[43].

4.2. Miscellaneous Metabolic and Physiological Functions

Apart from N acquisition and amino acid metabolism, various assorted physiological functions are known to depend directly or indirectly on σ^{54}.

rpoN mutants of *P. putida*[61] and *P. aeruginosa*[107] display multiple defects in their ability to use diverse C and N sources. Yet, some of these phenotypes are often difficult to trace to the loss of activity of specific promoters. Other metabolic and physiological functions, generally dispensable, are more clearly connected to the σ^{54} sigmulon. For instance, polyhydroxyalkanoate (PHA) accumulation from gluconate in *P. aeruginosa* required a functional *rpoN* gene. Both σ^{70} and σ^{54} promoters were found upstream of the operon encoding *phaC1Pa* (PHA synthase) and *phaDPa* (PHA depolymerase)[105]. Similarly, the *lipA* gene of *P. aeruginosa*, encoding a powerful lipase, depends on *rpoN* for expression[46], in combination with a cognate two-component regulatory system (*lipR/lipQ*)[92]. Another studied case of metabolic function subject to σ^{54} control is the gene encoding the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene of *Pseudomonas mevalonii*. This is an interesting enzyme, as it is homologous to a mammalian enzyme at the core of cholesterol biosynthesis (although it behaves as a catabolic enzyme in bacteria). The corresponding promoter includes a $-12/-24$ consensus[113] and 80 bp A/T rich region located 48–84 bp upstream from the transcription start and a regulator binds to it[112]. This AT-rich region appears to be the target of a regulatory protein. The proximity to the $-12/-24$ motif is unusual, and it cannot be ruled out that in fact such binding site results from a combination of σ^{54} and σ^{70} promoters. On the more general side, some C-starvation genes have been proposed to be regulated by *rpoN* in *P. putida*[57]. In fact, on the basis of genomic predictions, it has been suggested that σ^{54} mediates a general stress response in *Pseudomonas*. Quite on the contrary, experiments show that unlike σ^{S} (σ^{38}) the loss of σ^{54} does not impair in any significant extent the ability of *P. putida* to survive long-term starvation. *rpoN* mutants are indistinguishable from the wild type in respect to solvent tolerance, resistance to heat shock or sensitivity to hydrogen peroxide[17]. These data suggest that the loss of σ^{54} does not compromise bacterial endurance to gross types of environmental stress. Morevover, these results point out the limitations—if not the deception—of genomic predictions when confronted with experimental data[17]. On the contrary, other general functions might be connected also to *rpoN*: the *dnaA* gene of *P. putida* is transcribed through two promoters, one of which shows strong homology to σ^{54}-dependent promoters[45].

4.3. Metabolism of Hydrocarbons and Related Compounds

As mentioned above, the function of *rpoN* in *Pseudomonas* was first detected in experiments showing the dependence of the *Pu* promoter of the TOL plasmid on σ^{54}(see ref. [25]). Since then, a whole list of catabolic

systems for aromatic substrates have been identified in many *Pseudomonas* strains able to employ more or less complex aromatic hydrocarbons and related chemical species as C-sources. The manifold regulation of the TOL operons of plasmid $_p$WW0 of *P. putida* mt-2, two of which are *rpoN*-dependent, is discussed in the dedicated Chapter 18 of this book and will not be addressed here (excepting our remarks on *Pu*). The same applies to the second and somehow arche-typical catabolic pathway for phenol and methyl-phenols borne by the *Pseudomonas* sp. CF600. Expression of the *dmp* genes is regulated by the EBP named DmpR, which activates the cognate on σ^{54} promoter *Po*. Both the enzymology and the regulation of this system are discussed in detail in Chapters 16 and 18. But other, less studied systems for degradation of aromatics are dependent also on *rpoN*. We briefly mention below other catabolic promoters of *Pseudomonas* shown or suspect of belonging to the σ^{54}-dependent class.

A well-characterized σ^{54}-dependent degradative pathway is the one borne by a strain of *Pseudomonas azelaica* able to degrade 2-hydroxybiphenyl by virtue of the products of the *hbp* genes. Expression of *hbpCA* and *hbpD* is activated by the action of the EBP HbpR on the corresponding σ^{54}-promoters[48]. The *hbpC* promoter contains two groups of UAS for the HbpR regulator: one proximal (at approximately the standard upstream distance) and one far upstream. The role of the distalmost UASs in transcription is barely detectable but they become fully active when located in the place of the proximal UASs[47]. An interesting angle of this promoter/regulator pair is that 2-hydroxybiphenyl stimulates the binding of HbpR to the UAS[108]. Other regulators of this type can be considered mostly variants of the DmpR or the XylR proteins. For example, the *o*-cresol-responding EBP named TouR for expression of the *o*-xylene degradation pathway of *P. stutzeri* OX1 has just a few sequence differences with DmpR[3, 4], despite the fact that each regulator controls entirely different catabolic pathways. The same is true for the PhhR protein of a *P. putida* isolate able to degrade phenol and monomethylphenols through the action of the products of the *phh* operon[72]. Similar cases do exist with other systems for degradation of aromatic compounds. The toluene-3-monooxygenase from *Burkholderia pickettii* PKO1 and the toluene/benzene-2-monooxygenase from *Burkholderia* (*Pseudomonas*) sp. strain JS150 are enzymes which differ not only in catalytic specificity and substrate range but also in the arrangement and sequence of the genes within the operons that encode the enzymes, *tbuA1UBVA2C* and *tbmABCDEF*, respectively. The σ^{54}-dependent PtbuA1 and PtbmA promoters are activated by their cognate regulators, TbuT and TbmR, which respond strongly to toluene, benzene and chlorobenzenes in a fashion not unlike that of XylR[33]. Taken together, these results strongly suggest that σ^{54}-promoters and regulators of the XylR/DmpR family have been recruited along evolution to control a whole range of pathways involving initial ring mono- or dioxygenations[63]. But such *rpoN*-related biodegradative systems are

not limited to aromatics: the *bmo* (butane degradation) genes of *Pseudomonas butanovora* appear to be expressed through a putative σ^{54}-promoter[97]. Finally, expression of the 2-haloalkanoic acid dehalogenase gene of a large transposon borne by *P. putida* PP3 is controlled by the NifA-like regulator named DehR[106].

4.4. Plant Pathogenesis Determinants

Most studies on this issue have been made in virulent strains of *P. syringae*. That *rpoN* mutants stop being virulent is based on the fact that master pathogenicity factors are expressed through σ^{54}-dependent promoters. For instance, *rpoN* was essential for production of the phytotoxin coronatine in *P. syringae* pv *maulicola*. Coronatine is a disrupter of the plant-borne damage signal jasmonic acid, and its production by *Pseudomonas* enhances disease symptoms when the bacteria infect *Arabidopsis* leaves. Coronatine elicits also a hypersensitive response (HR) when *P. syringae* pv *maulicola* is infiltrated into tobacco, a non-host plant[40]. Yet, the main regulatory players of *P. syringae* virulence are the *hrpS, hrpL* and *hrpR* genes. *hprR* and *hrpS* encode σ^{54}-dependent regulators, while *hrpL* (encoding a member of the ECF subfamily of sigma factors) is expressed from a σ^{54}-dependent promoter. In *P. syringae* pv tomato the *avrD*[94] and *avrE*[66] loci for avirulence response (HR) are expressed through σ^{54} promoters and require both the *hrpSR* and *hrpL* regulatory genes[94]. This indicates that various σ^{54}-dependent steps are at play in the regulatory cascade leading to expression of virulence/avirulence[38]. That expression of *hprL* depend on *rpoN* has been observed as well in the pathovar *maulicola* of *P. syringae*[38], thus ultimately linking σ^{54} with the loss of the many virulence traits controlled by *hprL*. It is noteworthy that the *hrpR* and *hrpS* genes are co-expressed in the same operon in *P. syringae* and both encode σ^{54} activator proteins that lack the AB domains altogether. Both HrpR and HrpS proteins are needed for full expression of the *hprL* gene, perhaps by forming a stable heteromeric complex which act on the corresponding σ^{54} promoter. This has been proposed on the basis of elegant yeast two-hybrid and column-binding experiments[44].

4.5. Control of Human/Animal Virulence Factors by σ^{54}

That σ^{54} is important for expression of many pathogenicity-related traits of *P. aeruginosa* is simply indicated by the fact that an *rpoN* mutant was 100-fold less virulent in mice and also impaired in killing *C. elegans*[39]. Yet, the same mutation had no effect on the detrimental effect of *P. aeruginosa* infection in plant growth[39]. This suggests that while σ^{54} controls virulence factors

in both plant and animal pathogens, each niche has selected a distinct set of genes, that is, there are no universal master regulators for virulence factors.

Major assets for the virulence of *P. aeruginosa* in cystic fibrosis (CF) include the production of surfactants and alginate. The *rhlAB* operon encoding rhamnosyltransferase (for rhamnolipid production) contains putative σ^{54} and σ^{70} promoters upstream[79]. The control of this promoter is complex, since it responds also to the quorum-sensing stimulon. Still, production of alginate (the exopolymer that endows *P. aeruginosa* colonies with a distinctive mucoidity) is the result of one of the most intricate regulatory circuits known in the bacterial world. The whole system includes the interplay of many promoters, dedicated sigmas and anti-sigma proteins. This regulatory net is discussed in detail in Chapters 1 and 9 in this volume. We briefly mention here the subset of promoters/genes believed or proven to be dependent on *rpoN*.

The key genes for formation of alginate are named *algD* (encoding GDP mannose dehydrogenase) and *algC* (phosphomannomutase). These genes are located in separate loci in the chromosome of *P. aeruginosa*[121] and in *P. syringae*[80]. Both *algD* and *algC* are expressed through the action of the regulatory protein AlgR1 (the regulator element of a two-component signal transduction system), a binding sequence for which is present in both promoters. AlgR1 becomes active (i.e., phosphorylated) at high osmolarity, an event that increases the affinity for the target promoters. Transcription of *algC* is decreased in an *rpoN* mutant[122], suggesting a direct or indirect involvement of σ^{54}-dependent factor in *algC* expression. The *algD* gene is transcribed by the form of the RNAP containing the alternative ECF sigma factor AlgU (AlgT, σ22 or σ^{E}) along with AlgR1[58]. But in addition, expression of both *algR1* and *algD* is affected in an *rpoN* mutant[28]. In reality, the *algD* promoter is transcribed by both RNAPs containing σ^{E} or σ^{54}. The corresponding promoters do overlap, so the separate lack of either sigma leads to underexpression (but not loss) of *algD* expression. Most CF isolates are mutated in MucA (the anti-sigma factor for σ^{E}), leading to *algD* overexpression. But in other cases, an unknown mutation increases the *algD* expression that is dependent on σ^{54} and not on σ^{E}. Moreover, in CF isolates mutated in MucA, σ^{54} acts as a repressor[8]. This suggests the existence of at least two different lanes of conversion to mucoidity, one channelled through *algR1*/σ^{E} and a second pathway involving a σ^{54} system. Mutational up-regulation of either channel may lead to the mucoid phenotype. The regulator of the σ^{54} promoter of *algD* has not been inequivocally identified. AlgB is a response regulator of the NtrC family that is involved in *algD* expression in *P. aeruginosa*. Yet, attempts to show binding of AlgB to the *algD* promoter have not been successful[116].

P. syringae also produces alginate, but it seems that the regulation is quite different as, unlike *P. aeruginosa, algR1* is not required for *algD* transcription (the *algD* promoter lacks the consensus sequence recognized by

AlgR1). However, both the *algD* and *algR1* upstream regions in *P. syringae* contained the consensus sequence for σ^{22}, suggesting that *algU* (*algT*) is required for transcription of both genes. In any case, *algD* is not dependent on σ^{54} in this bacterium.

4.6. Fimbriae, Flagella and Other Envelope Proteins

Extracellular appendices are essential for a range of survival-related functions, including adhesion to surfaces and motility. A saturating transposon mutagenesis of *P. aeruginosa* followed by the screening for loss of fimbriae and impaired motion resulted in insertions clustered in three loci (*pilA, pilBCD* and *pilT*) determining the production of type IV fimbriae, as well as discrete insertions in *pilR, pilS* and *rpoN* genes. PilS shares homology with the protein kinase sensors of the two-component regulatory systems, while *pilR* is co-transcribed with *pilS* and might encode the NtrC-like regulator element of the same two-component pair[9, 41]. Expression of the pilin type IV gene (*pilA*) requires both σ^{54} and the PilS and PilR proteins. Ectopic transcription of *pilA* from the *Ptac* promoter in an *rpoN* strain results in piliated bacteria. This suggests that the role of σ^{54} is specific for *pilA* and does not control expression of any other gene necessary for pili formation[59]. The *rpoN* mutant of *P. aeruginosa* is non-piliated and shows diminished adhesion and internalization to human endothelial cells, a phenotype similar to the strain defective in the pilin structural gene[87].

Apart from type IV fimbriae, *rpoN* is required for the production and assembly of the flagellum. In this respect, the involvement of σ^{54} is not unique to *Pseudomonas*, since the same sigma participates in the formation of flagella in *Vibrio cholerae, Caulobacter crescentus, Vibrio parahaemolyticus* and *Campylobacter coli*[6]. In addition, some bacteria (*P. aeruginosa* among them) possess a dedicated sigma factor (σ^{28}) encoded by *fliA*, the activity of which is checked by the FlgM anti-sigma factor. The *flgM* gene is transcribed by both σ^{28} and σ^{54}(See ref. [32]). *fliC* (the structural gene for flagellin) is not expressed in *rpoN* mutants of *P. aeruginosa*[107]. Non-motile mutants in genes *fleS* and *fleR* correspond to genes encoding a two-component σ^{54} regulatory system, located upstream of operon *fliEFG*, which determines the flagellum basal body. FleS resemble histidine kinases which in other two-component systems have been shown to be sensor proteins, while FleR is homologous to other regulatory σ^{54}-dependent proteins. The *fleR* mutant is both non-motile and unable to adhere to mucin, thus suggesting that the regulator affects the expression of both the flagellum and other adhesins[91]. The *fleSR* operon is regulated by an additional σ^{54} regulator (FleQ), but the *fleQ* promoter is not itself dependent on *rpoN*[5]. FleQ is a σ^{54}-dependent activator placed at the top level of the flagellar hierarchy, as it regulates not only *fleSR* and *fliEFG*, but also

flhA and *fliLMNOPQ* encoding the flagellar export machinery[55]. Analysis of FleQ dependent promoters shows that the binding sites for this regulator are located not only upstream of the −12/−24 motifs, but also adjacent to the polymerase binding site. This implies an activation mechanism in which the regulator probably contacts the RNAP without looping.[55]. *fleQ* also activates *fliD*, encoding the filament cap protein (also called the hook-associated protein 2 or HAP2) that is implicated in mucin adhesion. The *fliD* gene might also be transcribed from a σ^{70} promoter[6]. Perhaps FliD makes the connection between production of flagella and mucin adhesion, both processes dependent on *rpoN*. It is interesting that pulmonary infection of *P. aeruginosa* selects *rpoN* mutants, as these are flagellum-deficient and thus they are internalized less efficiently by phagocytes[29]. It is noteworthy also that *rpoN* mutants of *P. putida* are non-motile and also lack flagella, the reason being the loss of expression of the *flhF* gene[76].

Finally, expression of some membrane-bound proteins appear to be connected to *rpoN*. For instance, the gene *oprE*, of *P. aeruginosa* PAO1, encoding an anaerobically induced channel-forming porin contains a putative σ^{54}-dependent promoter[117]. Interestingly, the lack of σ^{54} alters *oprE* transcription in aerobiosis but not in anaerobiosis. This gene is doubtless subject to a complex regulation, since the whole sequence 500 bp upstream of the transcription start, containing an IHF site and an AT rich region, is required for full *oprE* expression[118].

5. MODELLING THE *Pu* PROMOTER OF THE TOL PLASMID $_{P}$WW0

The regulation of the catabolic promoter *Pu* by its cognate regulator XylR and by various physiological controls is being discussed in various contexts in Chapters 13, 16, and 18 of this book and mentioned at several points along this article. In this section, we will underline a few emerging features of this promoter which highlight its status as a favourite experimental set-up for σ^{54} promoters.

Pu is activated at a distance by a toluene-responsive activator (XylR). This involves the binding of an oligomer of the regulator to UAS and the looping-out of the complex drawing it into close proximity to the σ^{54}-RNAP polymerase bound to the −12/−24 region. This event is assisted by IHF. The performance of *Pu* is exquisitely dependent on the metabolic status of the cell. An excess of certain carbon sources or rapid growth in rich medium inhibits the promoter in vivo even if toluene is present in the culture or if a constitutive XylR variant is employed. At least four different physiological inputs are channelled into the promoter. First, the presence of glucose and other carbohydrates controls *Pu* activity through a process which involves the *ptsN*

gene, encoding the IIANtr protein of the phosphoenolpyruvate:sugar PTS[20]. Second, rapid growth in a rich medium down-regulates the activity of *Pu* probably by keeping the performance of the σ^{54} protein under check, a phenomenon that has been termed *exponential silencing*[19]. Third, there seems to be a separate control connected to the heat-shock response, as revealed by the significant loss of activity of the promoter in cells defective in the FtsH protein[14]. Finally, *Pu* is moderately stimulated by the alarmone (p)ppGpp that mediates the stringent response to amino acids starvation[16]. None of these observations, however, explain entirely the phenomenal burst in activity of the promoter (>5000-fold) when *P. putida* cells pass from exponential growth to stationary phase. The exact role of (p)ppGpp in the transcription process is not known, but it has been suggested that the binding of this molecule destabilizes the RNAP–promoter open complex leading to escape of the transcription machinery and thus enhanced transcription[16]. Another possibility is that (p)ppGpp binding influences the outcome of sigma factor competition for the available core enzyme[62]. It is also possible that sigma factor competition is influenced by other ppGpp-independent, growth phase related phenomena. In addition, an anti-sigma factor specific for σ^{70} was found to be produced during the stationary phase in *E. coli*, and named Rsd, for regulator of σ^{D} [50, 51]. Finally, the system is further complicated by growth-phase dependence of intracellular XylR and IHF concentrations[31, 109] as well as by the temperature dependent effect of an additional factor named TurA[90b].

Since it is impossible to simultaneously control or accurately measure all parameters affecting this complex regulatory network, it is difficult to evaluate the significance of these various effects in the outcome of promoter activity. However, such a regulatory network can be approached with a dynamic mathematical model of σ^{54}-dependent catabolic promoters[110]. The model considers binding of the activator protein to the UAS, union of the sigma factor with the core polymerase, formation of the open complex, and escape of the transcription machinery from the promoter region, growth-phase control of IHF σ^{70} regulation during stationary phase, and the contribution of (p)ppGpp to both sigma factor selectivity and promoter escape. Interestingly, each combination of three of these effects predicted a similar behaviour of the promoter: repressed during exponential growth and sharp increase as the cells enter stationary phase. Although numerical data available for this system is scarce, the model has proved useful for interpreting the experimental observations and to evaluate the hypotheses that have been proposed to explain the phenomenon of physiological regulation. In any case, it appears that the mechanisms that couple *Pu* activity to the physiological status of the cells are both robust and redundant, thereby suggesting a considerable evolutionary drift towards an optimal interweaving of specific (i.e., effector-dependent) and general (physiological) signals.

6. CONCLUSION: SIGNAL INTEGRATION IN σ^{54}-DEPENDENT PROMOTERS

Why σ^{54} promoters and why so frequent in *Pseudomonas*? As discussed above, it appears that most *rpoN*-related functions involve traits that are required for survival or adaptation of cells to somehow harsh nutritional or environmental conditions. We advocate that what makes the difference between σ^{54} promoters and the others is their superior ability to integrate environmental signals[18]. In fact, the pivotal role of non-linear DNA allows these promoters to evolve signal integration devices which, to some extent, parallel the transduction cascades employed by higher organisms to control cell growth and differentiation. In general, regulatory cascades allow the possibility of inserting additional checks, either positive or negative, in every step of the process. DNA bending in σ^{54} promoters makes geometry the key regulatory element. In this way, σ^{54} promoters can include multiple metabolic control levels through just altering promoter architecture, so that positive signals favour an optimal constellation of protein–protein and protein–DNA contacts required for activation. Since, unlike eukaryotes (Figure 6), the number of channels to enter physiological signals in prokaryotic promoters is limited, bacteria seem to have thoroughly exploited the physical properties of DNA along evolution. Additional effects of regulated DNA bending in σ^{54} promoters include the possibility to amplify physiological signals and also to increase or decrease promoter specificity or promiscuity for cognate regulators, both aspects appearing to play a pivotal role in adaptation to novel environments[18, 85].

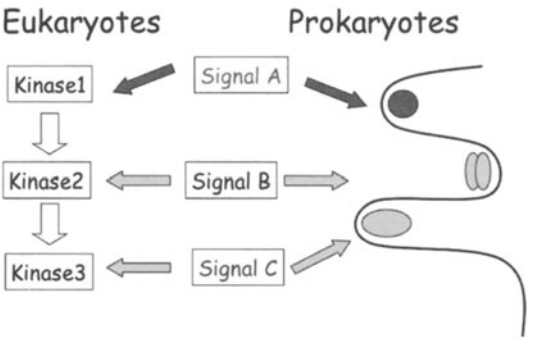

Figure 6. Signalling pathways in eukaryotes and prokaryotes. The sketch symbolizes the fact that eukaryotic transduction pathways generally exploit kinase cascades for signal integration, amplification and suppression of regulatory noise. On the contrary, prokaryotic promoters, in particular those depending on σ^{54} exploit intrinsic and protein-induced changes in DNA geometry for the same functions[85].

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11

COMPILING SIGMA-70-DEPENDENT PROMOTERS

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1. INTRODUCTION

Promoters are sequences that precede transcriptional start sites and determine recognition by RNA polymerases to initiate transcription. Most bacterial genes are regulated at the level of transcription, and expression from different classes of promoters is under the control of RNA polymerases with different sigma factors. The eubacterial core RNA polymerase is comprised of one β, one β′ and two α subunits[62]. Promoters are recognized by the holoenzyme (Eσ) formed by the reversible association of the core with a σ factor. This σ subunit confers specificity in promoter sequence recognition and is essential for transcription initiation[73]. Global switches in transcription patterns are known to be related to promoter selectivity of multiple RNA polymerases with different sigma factors[55], where the competition of sigma factors for core occupancy in each situation plays a key role[52, 78, 102]. Typically, bacteria have a major sigma factor that recognizes a large number of promoters, especially those controlling expression of the housekeeping genes. In Gram-negative bacteria, this factor is σ^{70}. Other specialized sigma factors such as σ^{28}, σ^{38}, σ^{32} and σ^{24} share high sequence homology with σ^{70} and are included in the so-called σ^{70} family[98]. The sigma factor σ^{54} constitutes a different group of its own, with characteristics that differ greatly from the σ^{70} family. The RNA polymerase with σ^{54} recognizes a specific subset of promoters with highly conserved sequences and architecture, completely different from other

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promoters, and where transcription initiation is always dependent on additional transcriptional factors[9] (de Lorenzo, Chapter 10 in this volume).

In both *Pseudomonas putida* and *Pseudomonas aeruginosa*, the genes for σ^{70}, σ^{38}, σ^{32}, σ^{28} and σ^{54} factors have been found and characterized[11, 30, 43, 72, 77, 104, 140, 161, 165, 166]. Moreover, multiple ORFs coding for putative extracytoplasmic function (ECF) sigma factors that constitute a phylogenetically and functionally distinct subgroup within the σ^{70} family have been found in their genome[130, 139]. Most members of this subgroup respond to signals from the extracytoplasmic environment. However, for many of them their specific function and target promoters remain unknown[106]. In both strains, at least five of these ECF sigma factors are organized in five analogous clusters that seem to be involved in iron uptake, a function that appears to be crucial for these strains. Some of the remaining *P. putida* ECF sigma factors seem specific and are related to the ability of this strain to colonize the rhizosphere, while *P. aeruginosa* has specific ECF sigma factors related to heme uptake and virulence associated secretion systems[106].

In this chapter we present a compilation of 149 σ^{70}-dependent *Pseudomonas* promoters in order to characterize the DNA sequence that is recognized by the σ^{70}-RNA polymerase in this genus. The promoters were selected after screening all *Pseudomonas* promoters published or recorded in available databases that had been defined as being σ^{70}-dependent and in which the transcriptional start point has been determined experimentally. We are aware that some of these promoters may require an alternative sigma factor of the σ^{70} family for transcription since strict σ^{70}-dependency is normally difficult to establish *in vitro*. However, the promoters selected for this analysis were originally reported to be σ^{70}-dependent, with the exception of Pm-pWW0 from *P. putida*, which depends on both σ^{32} and σ^{38}, but also shows significant basal activity *in vivo* with σ^{70} (Ref. 105). In the *P. aeruginosa* promoter *algC*, we also found a good $-12/-24$ consensus sequence for σ^{54}-dependent promoters. However the authors ruled out the possibility of this promoter being σ^{54}-dependent since it is regulated by AlgR1[187]. Therefore, this promoter has been included in the compilation as σ^{70}-dependent.

2. COMPILATION

2.1. Compilation Procedure

The promoters included in the compilation were obtained in three ways. A first set was selected after downloading from GenBank (release 135.0) all *Pseudomonas* sequences that included the word "promoter" within their annotations. Then the selected sequences were screened for the presence of an

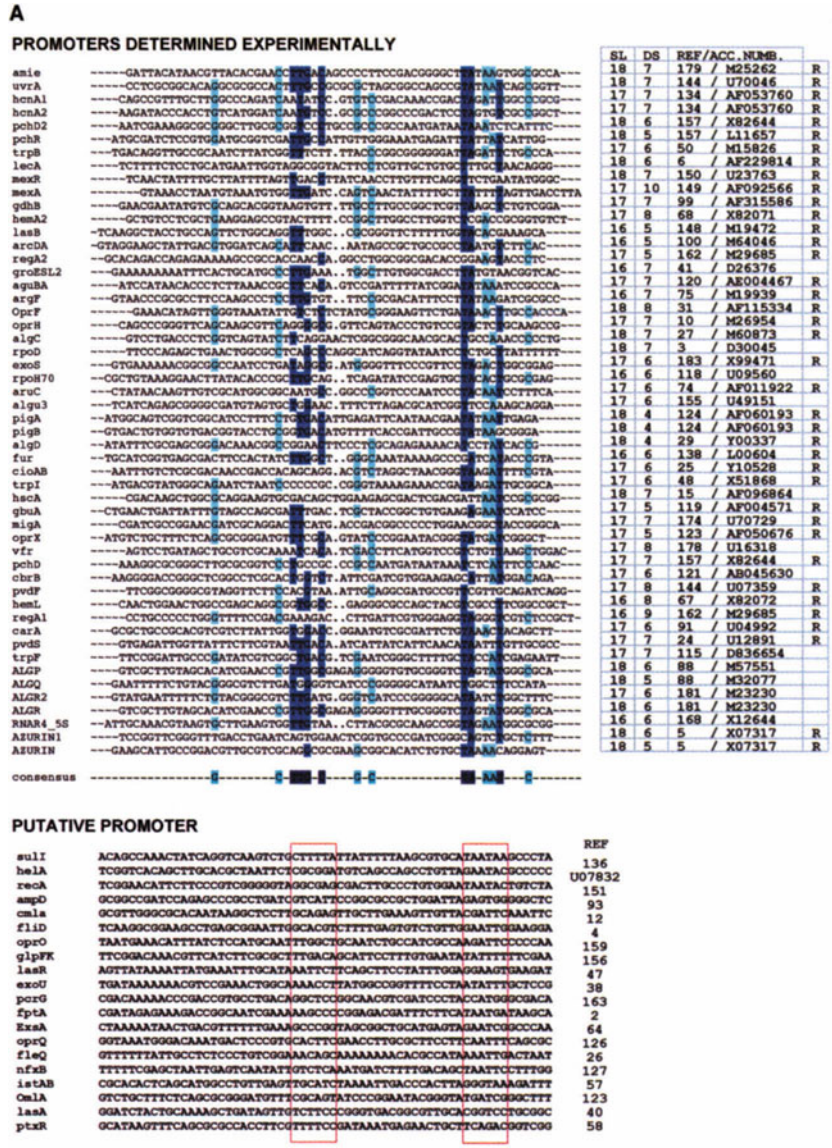

Figure 1. Continued

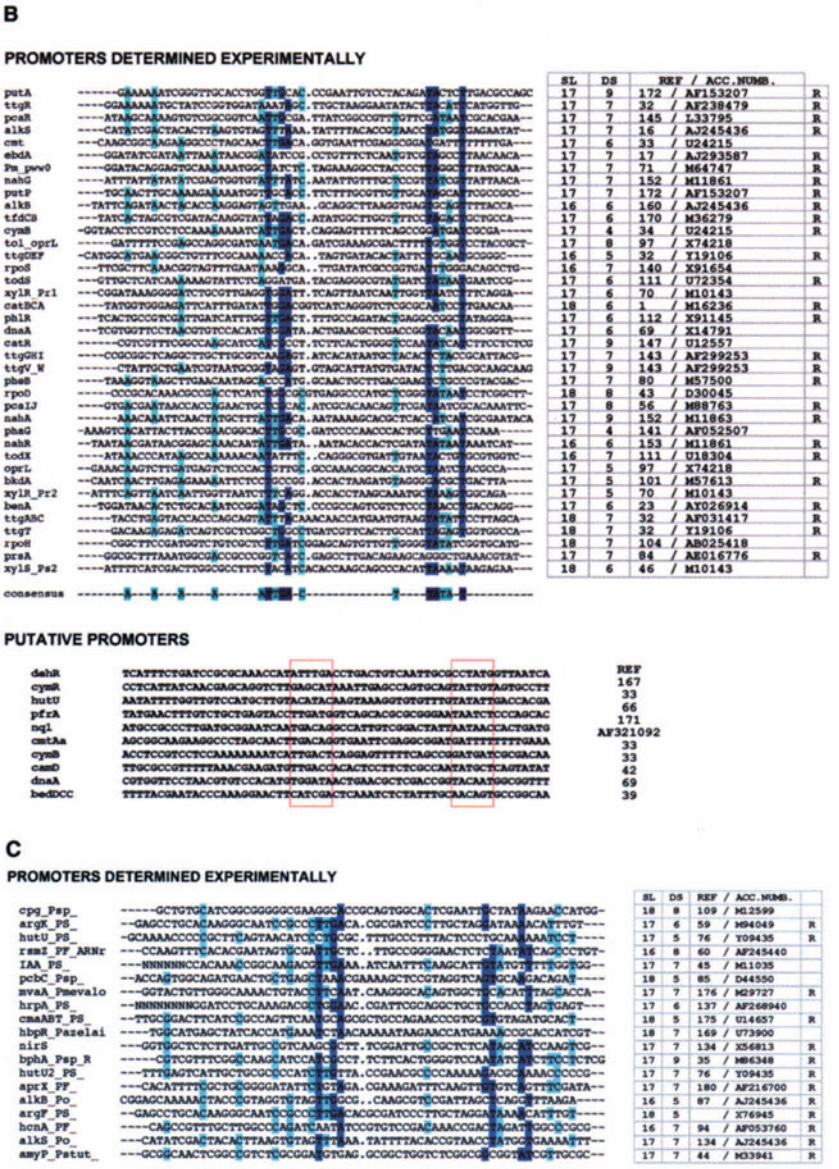

Figure 1. Continued

annotation for an mRNA start site. For each start site, the references provided were checked to verify that the transcription initiation site had actually been determined experimentally by primer extension analysis, S1 mapping or any other equivalent technique. A second subset of promoters was obtained by searching PubMed with the string "*Pseudomonas* AND promoter," which produced *c.* 1300 publications. Titles were then manually screened to locate those that included promoter analysis or description. When possible, the sequence provided was then confirmed in GenBank. Promoters from the two sets were combined and duplications were eliminated. Finally, a third set of promoters was obtained by screening GenBank for *Pseudomonas* promoters with annotations for a −10/−35 sequence, which could indicate the location of a putative promoter. Again, the references provided were used to determine the degree of confidence of the suggested RNA polymerase binding site. Those promoters presenting and experimentally identified transcription start site were included in the first set. The remaining suggested promoters were aligned separately as "putative promoters" (Figure 1 and see below).

2.2. Promoter Sequence Alignments

The sequences listed in Figure 1 are written in 5′ to 3′ orientation and start at position +1, that is, the first mRNA nucleotide (+1, right end), and extend up to position −60 (left end). Shorter fragments are presented when the complete sequence was not provided in the original publication. Sequences were first aligned using pairwise global alignment of all sequences, followed by progressive assembly of alignments using neighbor-joining phylogeny[131] as provided with the Align Plus 4 software version 4.10 included in the Clone

Figure 1. Continued
Compilation of *Pseudomonas* σ^{70}-dependent promoters. The left column gives the gene name for each promoter sequence. In the case of multiple start sites, promoters are designated P1, P2, etc. The sequences are written in 5′ to 3′ orientation. The first base on the left is position −60 and the last base on the right is the first base of the corresponding mRNA (position +1). The spacer length (SL), the distance to the transcription start site (DS), and the reference to the promoter sequence or to the determination of the start site (REF) are indicated to the right of each sequence. When no reference was available, the GenBank accession number is provided. An R in the last column indicates that the promoter is regulated by additional transcriptional regulators. As indicated, two alignments are presented for each species: an alignment of the experimentally determined promoters and a second alignment of putative promoters as described in the literature. (A) Alignment of *P. putida* promoters; (B) alignment of *P. aeruginosa* promoters; (C) alignment of promoters from other *Pseudomonas* species. PF is *P. fluorescens*, PS is *P. syringae*, Po is *P. oleovorans*, Pmevalo is *P. mevaloni*, Pazelai is *P. azelaica* and Pstut is *P. stutzeri*. The consensus sequence represents bases present in more than 50% (dark blue highlighted positions) or 40% (pale blue highlighted positions) of the sequences.

Manager Professional Suite package (Sci. Ed- Central). Alignment of the +1 position was forced by changing the alignment parameters as follows: open gap and extended gap penalties were set to 9, their maximum value, whereas the mismatch penalty was set to 0, its minimum value. The alignment obtained was saved in Rich Text Format and was further converted into MSF format using HMMER, an on-line version of SREFORMAT (www.pasteur.fr/seqanal/interfaces/sreformat.html). The identities in the alignment were highlighted using MacBoxShade v. 2.01 (M.D. Baron, Mercutio MDEF, 1997; http://iubio.bio.indiana.edu/soft/molbio/mac/) and a consensus sequence was extracted using the same program.

In a second step, visual inspection of the aligned sequences suggested that some of the sequences should be shifted one or two bases upstream or downstream, or a gap between the −10 and −35 region should be inserted or deleted, in order to increase sequence homology with the consensus. The alignment criteria hierarchy was as follows: (a) Conservation in the −10 region was preferred; (b) Distances of 10 nucleotides between the transcription start site and the −10 hexamer were avoided. However, for the *mexA* promoter of *P. aeruginosa*, we chose a 10 nucleotide distance from the −10 hexamer to the start site, which rendered the best alignment; (c) Spacer distance was manipulated to increase conservation in the −35 region, although deviation of more than 1 bp from the canonical 17 bp distance was avoided. A second alignment was obtained by forcing the parameters to conserve the manual displacement of the sequences, and was again depicted using MacBoxshade. The sequences presented in Figure 1 are ordered according to the phylogenetic data obtained after the second alignment. The consensus sequence represents bases present in more than 50% (dark blue highlighted positions) or 40% (pale blue highlighted positions) of the sequences.

Several alignments were attempted of the promoters grouped in different subsets. After aligning the sequences from the different *Pseudomonas* species both separately and as a whole, is was clear to us that differences existed between *P. putida* and *P. aeruginosa* promoter compilations. Therefore, two independent alignments are presented (Figures 1A and B respectively). The number of promoter sequences available for other *Pseudomonas* species was limited, thus sequences for the other *Pseudomonas* species are presented in a single alignment (Figure 1C) grouped by species. In this case, the alignment was optimized as above. In addition, 10, 25 and 9 putative σ^{70}-dependent promoters of *P. putida, P. aeruginosa* and other *Pseudomonas* species, respectively, have been aligned according to the suggested −35/−10 hexamers and are presented as separate alignments for each species. These putative promoters have not been considered in the analysis presented below.

3. ANALYSIS

The overall base composition of the aligned promoters 60 bp upstream from the +1 is 28% A, 26% T, 24% C and 22% G for *P. putida*, and 23% A, 26% T, 26% C and 25% G for *P. aeruginosa*. Thus, this region is significantly richer in AT pairs (54% and 49%, respectively) than the genomes from which they derive (38.4% and 33.4%, respectively). This is in agreement with data obtained from the compilation of σ^{70}-dependent promoters from *E. coli*[96, 129] and σ^{A}-dependent promoters from *Bacillus subtilis*[61]. The presence of AT-rich regions in the promoter sequences has been related to specific features of this region such as structural perturbations to enhance transcription[128] or target sites which interact with the RNA polymerase α-subunit C-terminal domain (αCTD)[50] or other cellular factors. The most conserved bases in the alignment for the *P. putida* and *P. aeruginosa* compilations were identified independently, but the results are very similar for both species. Overall, the pattern resembles the consensus described for *Escherichia coli* and *Bacillus subtilis*: two conserved hexamers centered at positions −10 and −35 relative to the transcription start site. However, although the −10 element in *P. aeruginosa* (**TA**t**A**A**T**) is similar to the *E. coli* consensus (**TATAAT**)[96], it is slightly different in *P. putida* (**TA**TAc**T**). On the other hand, the −35 element in *P. aeruginosa* (**TTGa**Cc) and *P. putida* (**TTGA**cC) is similar, though with a different degree of conservation, and slightly different from the *E. coli* consensus (**TTGACA**) (bases in bold uppercase letters and plain uppercase letters are present in more than 50% and 40% of the sequences, respectively, whereas bases in lower case letters are present in more than 30% of the sequences; Table 1). In *E. coli*, direct interactions between the nucleotides G and C of the −35 element and two arginine residues of the region 4.2 of σ^{70} have been described. These two residues are conserved in the two pseudomonad σ^{70} sigma factors. Altogether, the domains 2.4 and 4.2 of σ^{70}, which are known to interact with the −10/−35 elements in

Table 1. Consensus and degree of conservation of the −10 and −35 elements in the different species.

	−35						−10					
Consensus	**T**	**T**	**G**	**A**	**C**	**C/a**	**T**	**A**	**T**	**A**	**A**	**T**
P. aeruginosa	54	56	52	33	52	31/27	86	71	33	40	46	75
Consensus	**T**	**T**	**G**	**A**	**C**	**C**	**T**	**A**	**T**	**A**	**C/a**	**T**
P. putida	74	44	56	59	38	41	82	72	44	41	33/30	82
Consensus	**T**	**T**	**G**	**A**	**C**	**C/a**	**T**	**A**	**A**	**A/c**	**A**	**T**
Others	79	47	53	63	31	37/31	58	47	37	37/31	58	53

For each compilation, the degree of conservation (in %) of the consensus nucleotide is indicated. Bases in lower case letters are the second significantly more abundant bases in the corresponding position.

E. coli, are identical in both *P. putida* and *P. aeruginosa*, and almost identical to the same region in *E. coli* σ^{70}. Only the S607 residue downstream from region 4.2 in *P. aeruginosa* σ^{70} is a threonine in *P. putida* σ^{70} (Figure 2).

To better visualize the sequence conservation between the promoters and to illustrate the underlying variability of the region we obtained a logo plot of the alignments, as shown in Figures 1A and B. Plots were generated using the extended version[49] of the original sequence logo program[154] provided on-line at http://www.cbs.dtu.dk/~gorodkin/appl/slogo.html. The modified program allows for the presence of gaps in the alignment and incorporates the facility to set a prior nucleotide distribution for the computation of the sequence

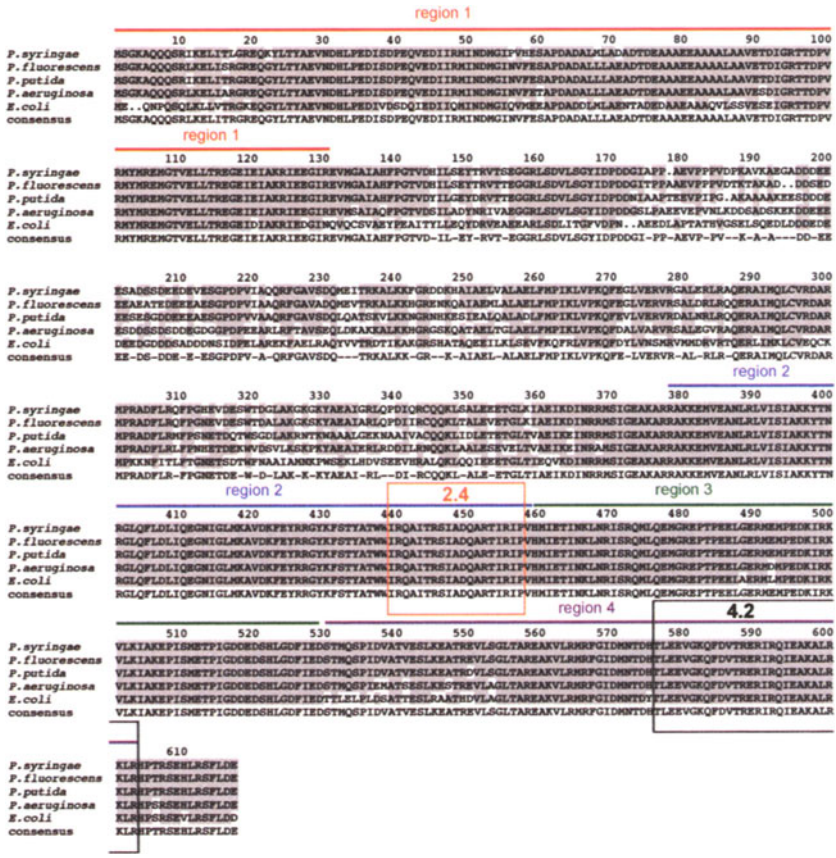

Figure 2. Alignment of σ^{70} from *E. coli, P. aeruginosa, P. putida, P. fluorescens* and *P. syringae*. Lines above the sequence indicate the functional regions as determined for *E. coli* σ^{70}. Specific regions 2.4 and 4.2, which are directly involved in DNA binding, are boxed.

information. In the logo plots presented in Figure 3A and B for *P. aeruginosa* and *P. putida* respectively, the prior base distribution was set according to the base composition of the 60 bp aligned sequence fragments, in order to emphasize the most significant bases within this specific region. Some interesting features can be observed in the results presented in Figure 3. In both species, the weight of the −10 hexamer is higher than that of the −35 hexamer (i.e., the total amount of Shannon information is higher in the −10 region). However, the −35 region in *P. putida* shows a better conservation than in *P. aeruginosa*, with a marked preference for T in the first position of the hexamer. Furthermore, the fourth and sixth positions of this hexamer are not clearly defined in *P. aeruginosa*. Upstream from the −35 region, a marked difference is observed between both species: there is a high frequency of As in *P. putida*, while Gs are found at a higher frequency in *P. aeruginosa*. It is worth noting that when the *a priori* base distribution was set based on the nucleotide composition of each genome, which is much higher in GC content (see above), the results were slightly different. Although the appearance of the −10 region was very similar, some differences were observed in the −35 region, where the weight of Gs and Cs was diminished for both species (not shown). In addition, the presence of As or Ts upstream from −35 was underscore, especially for *P. putida*.

Escherichia coli is rather tolerant to deviation from the consensus sequence of the double hexamer, as shown by the fact that on average only 7.1 out of the 12 bases are conserved in this species[96]. On average, promoters from both *P. putida* and *P. aeruginosa* match the consensus at half of the positions (*c.* 6.6 nucleotides out of 12 per promoter). In general, better matches are found in the −10 element, although this could reflect the criteria used to optimize the alignment, where matches in the −10 region were preferred. We should note that many of the promoters compiled are regulated by additional specific transcription factors (promoters marked with R in Figure 1). In *E. coli*, positive regulators of σ^{70} promoters generally bind close to the RNA polymerase binding site or even overlap this region. Thus, this could account for the poorer homology found in the −35 element. It is worth noting that we could not find a perfect match (12 out of 12) in any of the promoters analyzed.

In addition to the conservation in the −10 and −35 hexamers, we noticed that some other positions in the promoter sequences show a high degree of conservation (over 40%). Figure 1 shows that in addition to the −10/−35 elements, *P. putida* has a conserved T 5 bases upstream from the −10 element, and an A eight nucleotides upstream from the −35 element. The T 5 bases upstream from the −10 element also gives a good signal in the sequence logo (Figure 3B), whereas in this representation a conserved G appears 9 nucleotides upstream from the −35 element. In contrast, in *P. aeruginosa*, a conserved GCC sequence is found 12 nucleotides upstream

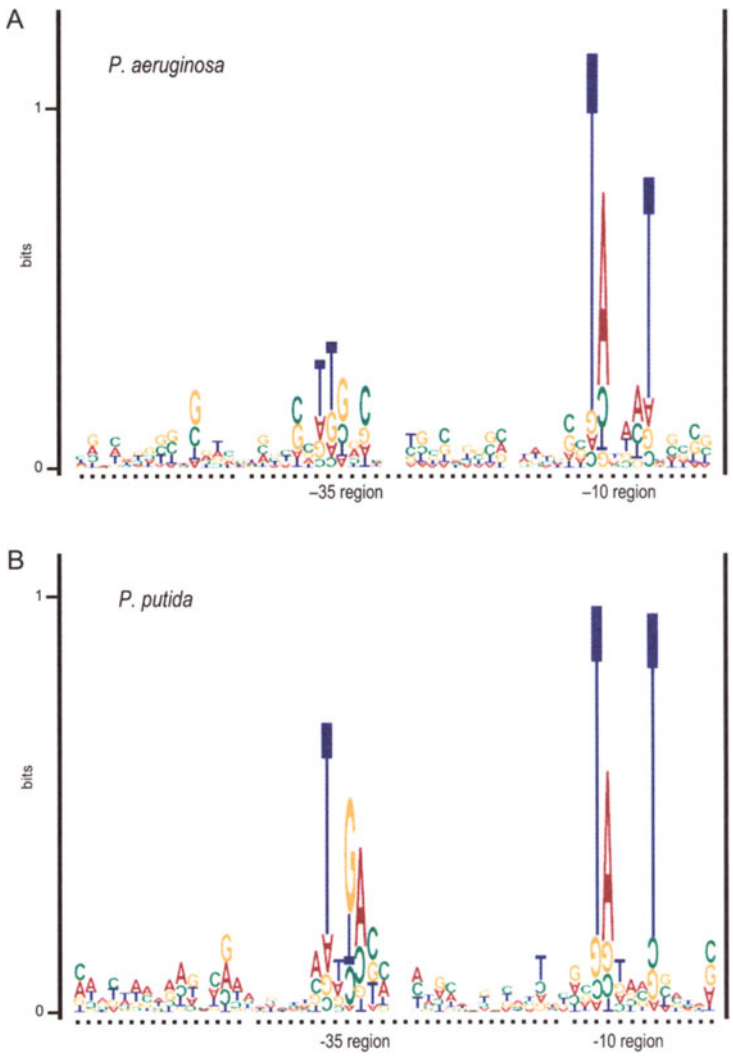

Figure 3. Sequence logo for the σ^{70} binding site in A. *P. aeruginosa* and B. *P. putida*. The height of the stack in each position is determined by the amount of Shannon information[22]. The height of each particular base symbol is proportional to its frequency. Bases underrepresented compared to their prior distribution appear up-side-down. The *a priori* base distribution used in these logos was 23% A, 26% T, 26% C and 25% G for *P. aeruginosa*, and 28% A, 26% T, 24% C and 22% G for *P. putida*.

from the −10 element (Figure 1A) and a conserved G 11 nucleotides upstream from the −35 element, which is also observed in the sequence logo (Figure 3A). These features have not been found in the alignments of either *E. coli* or *B. subtilis*. Recent analysis of the crystal structure of RNA polymerase from *Thermus aquaticus* and *E. coli* suggests that as yet unidentified additional elements may be recognized by the RNA polymerase in the process of initiating transcription[103, 114]. This may be the case for these conserved bases.

In *Pseudomonas*, the spacer sequence between the −10 and −35 elements has an average length of 17 bp, though a 1-bp deviation from this distance occurs with some frequency (Figure 4A), as is also the case in *E. coli*. However, flexibility in the spacer length is higher in *P. aeruginosa* promoters, where 50% of the promoters compiled deviate from the canonical distance, whereas in *P. putida* as many as 75% of the promoters have 17 bp between the −10/−35 hexamers. According to the recently determined crystal structure of *E. coli* σ^{70}, the structure may allow recognition of promoters with spacer lengths ranging from 16 to 18 bp by kinking or bending DNA to bring the elements into an optimal conformation[65]. The average distance between the −10 element and the transcription start site is 6–7 bp in both species, although values between 4 and 10 have been measured (Figure 4B). As is the case for other species, the preferred base for transcription initiation in *P. putida* is adenine, present at position +1 in 50% of the promoters. Surprisingly, no start site base preference is observed in *P. aeruginosa*. It is worth noting that the GC content in the region between the −10 hexamer and the transcription start site was slightly different in *P. aeruginosa* (62%) and *P. putida* (57%).

We searched the compilation for the presence of conserved bases upstream from the −10 element. In *E. coli*, a group of promoters known as "extended −10" promoters lack a conserved −35 element but present the dinucleotide TG at positions −15, −14. A role in promoter melting has been attributed to this new element, which makes contact with specific positions of the σ^{70} region 3.0 (previously named 2.5)[8]. In the *B. subtilis* compilation, *c.* 50% of the promoters contained this element[61]. In contrast, neither T nor G are present at above average frequencies in the −16 to −13 region of the promoters aligned in Figure 1, and only 6 out of the 110 promoters aligned in this analysis had a TG sequence 1 base upstream of the −10 element. Thus, at least in the promoters included in this analysis, this element does not seem to play an important role.

We searched for the presence of AT tracks in different regions of the promoters. In both *P. putida* and *P. aeruginosa*, 40% of the promoters contain one or more tracks of As or Ts of 4 bp or longer. However, the distribution of these tracks is different in the two species. In *P. aeruginosa*, these elements are uniformly distributed between the spacer region and the region upstream from

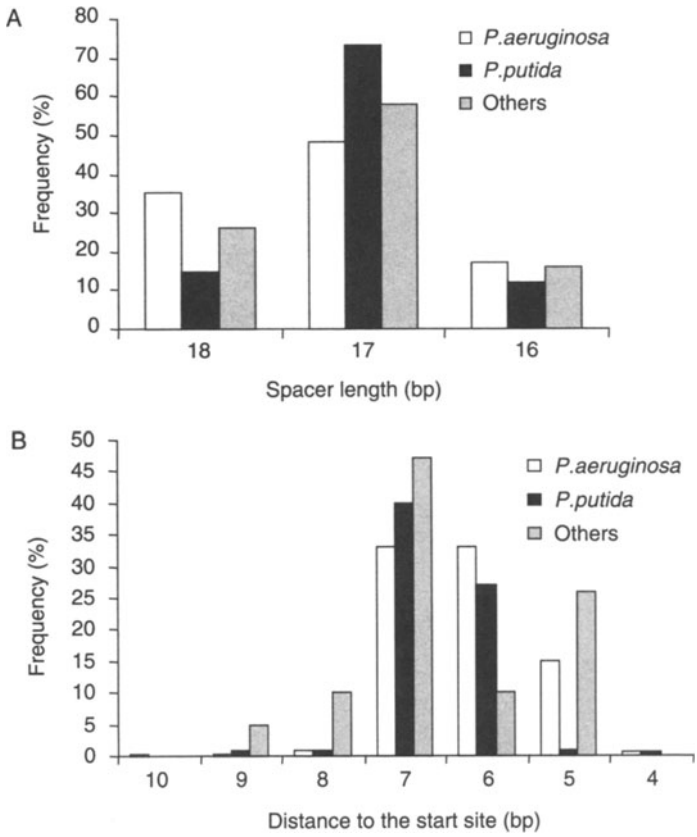

Figure 4. A. Distribution of spacer length for the experimentally determined promoters aligned in Figure 1. Spacer lengths were assigned by optimizing match to the consensus for the −10 and the −35 regions (see text). B. Distribution of the mapped start sites as a function of distance from the last base of the −10 element.

−35 (Figure 5), and their preferred length is 4 bases, whereas in *P. putida*, a similar number of 4-, 5- and 6-bp-long tracks are found mostly upstream from the −35 hexamer (Figure 5B). It has been suggested that these elements create a specific three-dimensional structure, and many authors have discussed the possibility that DNA bending in this region could enhance promoter activity (ref. [128] and references therein). In certain *E. coli* promoters, such as *rrnB* P1, AT tracks forming the so-called UP element are known to interact with the RNA polymerase α subunit[146]. In the *B. subtilis* promoter compilation, overrepresentation of alternating A and T tracks in the −36 to −80 region was observed, which led the author to predict that an upstream region with this

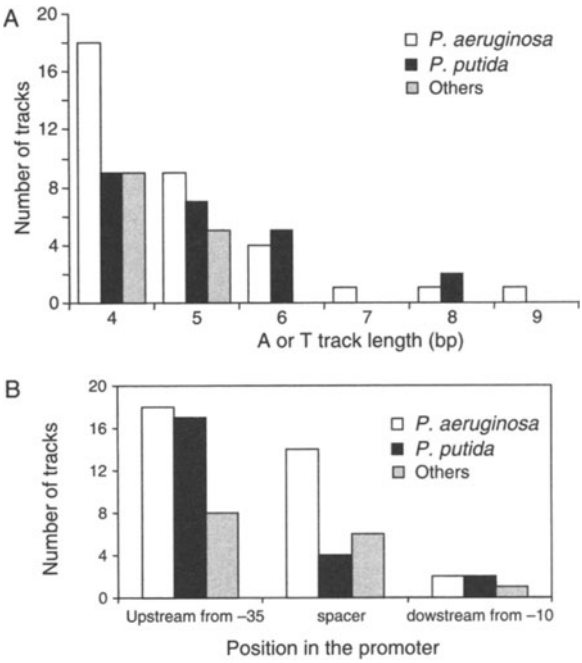

Figure 5. A. Occurrence of A or T tracks of different lengths. The number of A_n o T_n was plotted against *n*. B. Enumeration of A or T tracks present in different positions of the promoters. "Spacer" indicates the region between the −10 and −35 elements. In this analysis, both experimentally determined and putative promoters of each group of organisms have been considered.

pattern might be stimulatory for transcription[61]. From the published data characterizing the activity of the *P. putida* promoters that have A-tracks upstream from the −35 region, we deduce that in most of the cases, these promoters are regulated. Therefore we suggest that these elements may be associated with binding of regulatory proteins.

4. CONCLUDING REMARKS

Here we present a compilation of the currently characterized *Pseudomonas* promoters and the derivation of a consensus sequence, which resembles the −10/−35 consensus of other genus. This is in agreement with the fact that σ^{70} from *E. coli* and *Pseudomonas* are almost identical in the 2.4 and 4.2 regions (Figure 2). It is worth noting that all the *Pseudomonas* σ^{70}-dependent promoters assayed to date are active in *E. coli*, and when necessary,

E. coli RNA polymerase responds to activation–repression mechanisms provided that the corresponding regulatory gene is also present. Thus, *E. coli* RNA polymerase is perfectly able to recognize and transcribe *Pseudomonas* promoters. However, the compilation reveals that some positions outside from this region are also highly conserved in *Pseudomonas* promoters, but are different between different *Pseudomonas* species. Although this observation is interesting, no specific function can be assigned to these sequences until experimental data are available.

In general, we observed that the presence or absence of a particular sequence element in a promoter or the degree of deviation from the consensus did not show strong correlation with the presence or fidelity of any other element, that is, conserved hexamers, A/T tracks, spacer distance or distance to the transcription start site. This indicates that probably all combinations of recognition elements are possible in the σ^{70}-dependent promoter structure, and strengthens the evidence in support of the currently proposed model for promoter activation, in which alternative pathways are possible for the RNA polymerase activation mechanism that are "identified" by the enzyme at every subsequent stage[103, 114].

Altogether, the information content of the consensus sequence obtained is not sufficient to allow the accurate automatic prediction of σ^{70}-dependent promoters in *Pseudomonas*. In spite of this, we found a good correlation between the consensus derived from experimentally defined promoters and the putative promoters identified by different groups (Figure 1). The consensus derived from the collection of 110 experimentally defined promoters shows a high degree of flexibility both in structure and sequence. This will make it difficult to generate efficient promoter-site-searching algorithms based on statistical weighing of a consensus base pair to search for and predict the presence of sequences with promoter activity in the growing number of sequenced pseudomonad genomes.

We should take into account that the results obtained from this compilation and the differences between the *Pseudomonas* species might be biased. *P. aeruginosa* has been studied in greater detail because of its implication in pathogenesis, so most of the genes analyzed and published to date are related with this characteristic, whereas *P. putida* is well known for its catabolic capacities. Many of the genes and promoters analyzed in the literature are involved directly or indirectly in the biodegradation of aromatic compounds. In many cases, it is well known that both types of properties are acquired by many bacterial strains through horizontal transfer; therefore some strains bear certain characteristics that may not be intrinsic to the host species itself, *Pseudomonas* in this case. However, it is also true that many of the genes compiled here are unrelated to these two functions, and were compatible with the reported consensus. Finally, it is worth noting that *P. putida* promoters are functional in *P. aeruginosa* and *P. aeruginosa* is functional in *P. putida*.

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12

COMPILING EXTRACYTOPLASMIC FUNCTION (ECF) SIGMA FACTORS REGULATED PROMOTERS IN *PSEUDOMONAS*

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1. INTRODUCTION

The regulation of gene expression makes it possible for a bacterium to effectively adapt to rapid changes in the surrounding environment. In bacteria there are several regulatory mechanisms during transcription and translation, the primary checkpoint being initiation of transcription in which the DNA dependent RNA polymerase is the key enzyme. Core RNA polymerase is a complex multisubunit enzyme that can associate in a reversible way with a sigma (σ) subunit forming a holoenzyme. Most bacterial species synthesize a number of different σ factors which direct the RNA polymerase core enzyme to distinct classes of promoters with a different consensus DNA sequence. Most transcription in fast growing bacteria is initiated by the primary σ subunit similar to the housekeeping σ^{70} of *Escherichia coli* which also often relies on DNA binding activator and repressor proteins for initiating transcription. In other cases however, the primary σ subunit is replaced by an alternative sigma factor with different promoter selectivity. In fact, the use of alternative sigma

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factors that alter RNA polymerase core specifity is a very simple and effective mechanism employed by bacteria to bring about a major switch in gene expression[15].

Bacterial σ factors are grouped into two large unrelated families, the σ^{70} and the σ^{54} family, the majority belonging to the former family[13, 42]. The σ^{70} family is divided into three structurally and often functionally related subgroups: (a) the subgroup 1 factors include σ^{70} family homologues responsible for most of the transcription in actively growing cells recognizing promoters of similar sequence, that is, TTGACA near −35 and TATAAT near the −10 region, (b) the subgroup 2 factors are similar to the primary group 1 σ factors but are non-essential for cell growth, one of the most studied example is the stationary phase sigma factor σ^{S}, and (c) the subgroup 3 factors are the alternative sigma factors which control transcription initiation during special or developmental conditions. This last group usually functions as global regulators allowing the coordinated regulation of many operons and is further divided into several clusters of evolutionarily related proteins with conserved functions. There are four major clusters of sigma factors in this group being the sporulation, flagellar, heat shock and the *e*xtra*c*ytoplamsic *f*unction (ECF) cluster. It has been determined that within these clusters in some cases the σ factors recognize conserved promoter sequences in diverse bacteria. A sigma factor and the genes under its specific control is called a regulon and sigma factor regulons have been mainly studied in *E. coli* and *Bacillus subtilis* and recently also addressed in *Streptomyces, Mycobacterium* and *Pseudomonas*. This chapter will focus on the *Pseudomonas* ECF σ factor cluster with a special emphasis on their target gene promoters.

1.1. The ECF Sigma Factor Family

Initially, several ECF sigma factors were identified and believed to act as classical positive transcriptional regulators. However studies in 1994 on the regulation of an extracellular agarase gene in *Streptomyces coelicolor* led scientists to the identification of a protein, now designated σ^{E}, which displayed sequence similarity to seven regulatory proteins from other bacteria. These eight proteins (SigE from *S. coelicolor*, CarQ from *Myxococcus xanthus*, AlgU of *Pseudomonas aeruginosa*, HrpL of *Pseudomonas syringae*, SigE and FecI of *E. coli* and SigX of *B. subtilis*) were proposed based on their similarity to σ^{70}, to constitute a subfamily of eubacterial RNA polymerases termed ECF sigma factors because they all appeared to regulate extracytoplamsic functions[19]. A sequence alignment of these eight proteins with representative members of the σ^{70} family revealed that they share similarity across two (regions 2 and 4) of the four conserved regions of σ^{70} proteins (discussed later).

The advent of completely sequenced bacterial genomes revealed a wide range in the numbers of ECF σ factors present in bacteria, for example, 2 in *E. coli*, 7 in *B. subtilis*, 10 in *Mycobacterium tuberculosis*, 19 in *P. aeruginosa* and *P. putida* and approximately 50 in *S. coelicolor*; ECF σ factors in most species are by far the most abundant group[13, 26]. Variation in sigma factor content indicates immense flexibility of transcriptional regulation in individual species and it is reasonable to speculate that a species which adapted to a constant environment has a smaller repertoire of alternative σ factors than a species which is forced to cope with many changing environmental conditions. It appears that many ECFs within a species evolved by gene duplication and that gene regulation controlled by such specialized ECF factors was a late evolving phenomenon[26].

Studies on numerous ECF σ factors have highlighted several common regulatory features which however are not universal. First, these factors usually coordinate transcriptional response to extracellular signals. Second, in most cases immediately adjacent to the sigma factor gene there is a gene encoding an anti-σ factor, typically membrane localized that binds and stochiometrically inactivates the ECF σ factor[13, 20, 31]. The transmembrane localization of the anti-σ factor consent the bacteria to monitor the activity of the intracellular ECF factor in response to signals outside the inner membrane (for examples see ref. [20]). Finally, very often ECF σ factors are under positive autoregulation which serves to amplify the signal once the active σ factor is released from the inactive σ–anti-σ complex. Recently, due to the rather large numbers of ECF present and because of these common features it has been proposed that the ECF family makes up a distinct group of sigma factors within the σ^{70} family known as "subgroup 4"[13].

1.2. ECF σ Factors in *Pseudomonas*

ECF σ factors have been extensively studied in *Pseudomonas* where they control several iron uptake pathways, alginate biosynthesis, expression of virulence factors, tolerance to several stresses, expression of a protease and of an outer-membrane porin[3, 5, 6, 31, 32, 37–39]. In *Pseudomonas*, two ECF systems have been best characterized and are both of *P. aeruginosa*; first, the σ^{E} (also known as AlgU and AlgT) regulator of alginate biosynthesis and other stress responses and second, σ^{PvdS}, a regulator of pyoverdine siderophore biosynthesis, of exotoxin A and of an endoprotease. A summary of the characterized ECF σ factors of *Pseudomonas* is presented in Table 1.

1.2.1. The σ^{E} ECF Factor of P. aeruginosa

The σ^{E} ECF factor of *P. aeruginosa* is required for transcription of alginate biosynthetic genes that are responsible for the synthesis of the exopolysaccharide alginate which confers a mucoid phenotype. This phenotype conversion

Table 1. ECF σ factors investigated in *Pseudomonas*.

ECF	Organism	Identified target gene(s)	−35 and −10 gene promoter consensus binding site	References
σ^E	*P. aeruginosa*	*rpoH, algU,R,D, oprF, lptA,B, slyB, osmC, asmA6,B2, C1,D3* etc. (see Table 2)	GAACTT—16/17 bp—TCcaA	[10]
σ^E	*P. fluorescens*	*algU*	Currently unknown	[32]
σ^{PvdS}	*P. aeruginosa*	*pvdA,D,E,F,R,Y, ptxR* etc. (see Table 3)	TAAAT—16 bp—CGT	[28], [41]
σ^{PfrI}	*P. putida*	*ppsD*	Currently unknown	[9], [37]
σ^{PbrA}	*P. fluorescens*	*sidA, pbsC, pbuA*	GAACTG—16 p—CT	[34]
σ^{PsbS}	*P. fluorescens*	*psbA*	Currently unknown	[1]
σ^{FpvI}	*P. aeruginosa*	*fpvA*	Currently unknown	[3], [30]
σ^{PupI}	*P. putida*	*pupB*	Currently unknown	[16]
σ^{SigX}	*P. fluorescens*	*oprF*	GAAGTT—16 bp—GTG	[5]
σ^{PrtI}	*P. fluorescens*	*aprX*	TGTAGA—16 bp—GTG	[6]
σ^{HrpL}	*P. syringae*	*avr* genes, *hrp* genes[43]	tGGAAt—16 bp—CCAC	[43]

plays a major role in the pathogenesis of *P. aeruginosa* lung infections of cystic fibrosis patients[12]. σ^E also directs expression of a gene encoding the major heat shock sigma factor σ^{RpoH} thus it is likely to play a role in global gene regulation[33]. In fact σ^E has also been recently shown to direct RNA polymerase to genetic loci encoding lipoproteins which are believed to be correlated with inflammation and clinical deterioration of mucoid strains in fibrocystic lung disease[10]. The σ^E ECF factor of *P. aeruginosa* is encoded by the *algU* gene which is part of the *algU-mucA-mucB-mucC* gene cluster which is very similar to the *rpoE-rseA-rseB-rseC* of *E. coli*. The MucABC proteins ensure tight control of σ^E activity in response to environmental stress conditions which have yet to be precisely deciphered. σ^E activity is controlled by the membrane localized MucA anti-σ factor either by targeting it for proteolysis, by destabilizing it or simply by sequestering it at the membrane. The MucA protein appears to be stabilized by the periplasmic protein MucB while the precise function of MucC is still unknown[4]. Recently, σ^E has also been reported in a plant growth-promoting *Pseudomonas fluorescens* strain and shown to be important for tolerance to osmotic and dessication stress but unlike in *P. aeruginosa*, it did not play a role in protection against heat and oxidative damage[32].

1.2.2. The σ^{PvdS} ECF Factor of P. aeruginosa

The σ^{PvdS} ECF factor of *P. aeruginosa* initiates transcription of genes required for the biosynthesis of the siderophore (iron transport agent) pyoverdine,

as well as for secreted proteins exotoxin A and PrpL endoprotease[3, 17]. The signalling pathway leading to σ^{PvdS} activation involves the ferri-pyoverdine outer-membrane receptor FpvA which upon interaction with ferric-pyoverdine transmits a signal to the anti-σ^{PvdS} factor FpvR which then releases the suppression of σ^{PvdS} resulting in the initiation of transcription of *toxA, prpL* and pyoverdine biosynthesis genes[17]. Interestingly, the ferric-pyoverdine/FpvA receptor/FpvR anti-σ factor cascade also controls the release of another ECF sigma factor (FpvI) which controls the expression of the *fpvA* gene[3]. This cascade in *P. aeruginosa* occurs only under iron-limiting conditions since the *pvdS* and *fpvI/fpvR* loci are negatively regulated by the iron-binding Fur repressor which blocks their transcription in the presence of excess iron. On the other hand, under iron-limiting conditions, ferric-pyoverdine initiates the cascade through FpvA and FpvR resulting in the release of the two sigma factors (σ^{PvdS} and σ^{FpvI}). Orthologues of the σ^{PvdS} ECF factor have been identified and shown to regulate siderophore production in *P. putida* (σ^{PfrI})[37, 38] and *P. fluorescens* (σ^{PbrA})[34], however the precise regulatory cascades have yet to be elucidated. A similar three-component system to the FpvA/FpvR/σ^{PvdS} has been elucidated in *P. putida* which regulates the transcription of an outer-membrane receptor gene responsible for the uptake of a heterologous ferric-siderophore. More precisely, under iron-limiting conditions and in the presence of the heterologous siderophore BN7 produced by a different *Pseudomonas* strain, ferric-BN7 interacts with the outer-membrane receptor protein PupB of *P. putida* which signals the anti-σ factor PupR resulting in the release of σ^{PupI} which directs more transcription of the *pupB* receptor gene[16]. Based on genome sequence analysis, it appears that *P. aeruginosa* and *P. putida* have several of these three-component systems in order to acquire iron from heterologous siderophore systems making these species highly evolved and competitive for iron assimilation[25, 39].

1.2.3. Other ECF σ Factors of Pseudomonas

Two other *Pseudomonas* ECF sigma factors have been characterized for their role in regulating gene expression. First, in *P. fluorescens*, the temperature dependent production of an extracellular protease, encoded by the *aprX* gene, is under the positive control of σ^{PrtI} and of a novel transmembrane activator protein (PrtR) which has little homology to anti-σ factors[6]. Interestingly, σ^{PrtI} specifically requires active PrtR to form a functional RNA polymerase. Another ECF factor recently identified in *P. fluorescens* and *P. aeruginosa* is σ^{SigX} which is involved in the regulation of a major outer-membrane non-specific porin (OprF) that plays a role in safeguarding cell shape; σ^{SigX} is also required for growth under low-osmolarity environments[5].

The number of studies involving ECF factors is likely to dramatically increase in the near future since the genome sequence of *P. aeruginosa*[36] and of *P. putida*[27] have revealed the presence of 19 ECF σ factors in each genome,

the second highest number so far with only *S. coelicolor* having more. In both species, 14/19 factors are likely to be involved in iron uptake and are phylogenetically clustered together and are refereed as "iron starvation (IS) sigmas"[25, 39]. It is believed that *P. putida* and *P. aeruginosa* are equipped with a series of iron capturing systems making these species competitive in the environments that they grow in. *Pseudomonas putida* strains are efficient rhizosphere colonizers and often are plant growth-promoting bacteria; also thanks to their iron monopolization which makes iron less available to plant-pathogenic microorganisms. *P. aeruginosa* strains on the other hand, have several virulence associated systems which are related to iron since it is an important signal in the regulation of pathogenic factors. The remaining ECF factors have homologues in a wide range of bacteria and are probably involved in response to various environmental stress factors[25, 39].

2. ECF σ FACTOR REGULATED GENE PROMOTERS

Sequence analysis of the ECF factors revealed that they retain many of the four conserved domains of σ^{70} factors, however they show significant differences from other members of the family[19] (Figure 1). Differences are most evident in region 1, which prevents direct binding of free σ to the promoter,

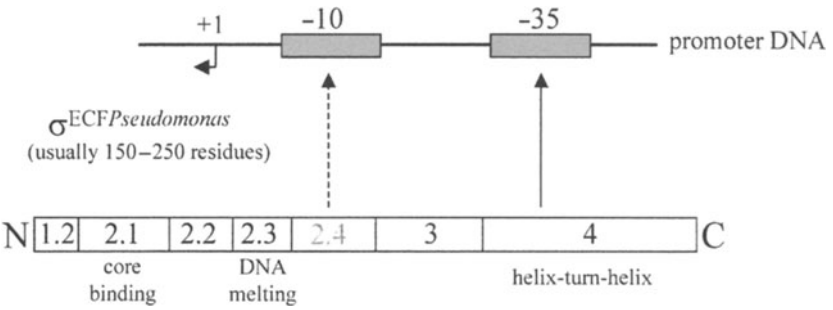

Figure 1. Conserved regions of σ^{ECF}s. Shown are the domains, which are conserved with members of the σ^{70} family. Domain 1, which has been implicated in preventing free σ from binding to the promoter, is absent in ECF factors (in some ECF factors, only a few residues are conserved in region 1). Domain 2 is the most conserved and contains regions implicated in core binding, DNA melting and interaction with the −10 promoter region. The region 2.4 is shaded and arrows are dotted as there is a very low degree of conservation in this region with the σ^{70} family. It appears however that it is conserved within groups of closely related ECF sigma factors[39]. Domain 3 has a very low degree of conservation (ECF factors are rather small and have lost most of this domain) whereas domain 4 is well conserved containing the helix–turn–helix DNA binding motif, which interacts with the −35 promoter region. See text for further details.

region 2.4, which interacts with the −10 promoter region, and region 3, whose precise function is unknown. Region 2 is the most conserved segment among σ^{70} family members analysed so far, it includes core binding determinants (region 2.1), DNA melting determinants (region 2.3) and residues implicated in interaction with the −10 promoter region (region 2.4). Interestingly, the characteristic feature of region 2.4 is a set of hydrophobic residues with proper spacing to form an amphipathic α-helix which is proposed to contain the −10 promoter sequence determinants; in ECF σ factors this 2.4 region is missing. Domain 4 is involved in −35 promoter sequence recognition and the ECF class displays the highest resemblance to other members of the σ^{70} family in this region, including the amphipathic helix (region 4.1) and helix-turn-helix DNA-binding motif (region 4.2). ECF members are not exceptional with respect to the requirement of two separate DNA-binding sites for promoter binding which are located in the −35 and −10 regions with respect to the initiation of transcription site. The studies of several ECF regulons from various bacteria including *E. coli, B. subtilis, Mycobacterium, Pseudomonas* and *S. coelicolor*, have revealed that there is some sequence conservation in the −35 sequence, whereas there is no real similarity in the −10 sequences which can be explained by the low degree of resemblance in region 2.4 in the members of the ECF class of factors[26]. This diversity of −10 promoter sequences and region 2.4 of ECF factors probably accounts for the coexistence of multiple members of the ECF family in the same species avoiding overlap in their biological functions.

Assigning the function to an ECF factor, identification of the target gene(s) and determination of promoter selectivity will be a great challenge for scientists in the future due to the recent discovery that several bacterial species have such a large number of ECF factors. Scientists have made important progress in this field in *B. subtilis* as three ECF sigma regulons (now also referred to as "sigmulons") have been extensively studied identifying target genes using several approaches and results are beginning to highlight how a species copes with having several ECF factors[13]. Also in *Pseudomonas*, studies on the σ^{E} and σ^{PvdS} regulons are beginning to give some insight in this rather novel field.

2.1. Strategies for the Identification of Target Genes of an ECF Regulon

When studying a specific σ^{ECF} regulon, the questions to answer are: How many genes are activated, what is the identity of these genes and which is the gene promoter selectivity. Recently several ECF sigma regulons have been, at large, studied using a variety of approaches. For example, using genetic and molecular methods, 20 target gene promoters of the *E. coli* heat shock ECF σ^{E} have been identified which activated gene transcription in a σ^{E}-dependent

manner[8]. An extensive analysis using transcriptional profiling microarray analysis, run-off transcription/macroarray analysis and comparative genome analysis promoter consensus search of the alkali induced σ^W of *B. subtilis* identified 30 target gene promoters[7]. Briefly, (a) transcriptional profiling uses a cDNA microarray allowing the simultaneous monitoring of mRNA isolated from wild-type cells compared to ECF mutant strain cells, aimed at identifying transcripts present in the wild type and reduced or missing in the ECF mutant; (b) transcription/macroarray analysis involves in vitro transcription (containing radiolabelled nucleotides) of digested chromosomal DNA with core RNA polymerase containing either the housekeeping σ^{70} factor or the ECF. The resulting ^{32}P labelled RNA populations are then hybridized to macroarray membranes allowing the detection of transcriptional effects that are directly linked to a specific holoenzyme; and (c) a consensus-based promoter search allows the rapid identification of candidate target genes based on their association with the specific target sequence recognized by the ECF factor. Thanks to a computer promoter consensus search, more than a dozen of target genes were identified for the σ^X ECF of *B. subtilis* highlighting its role in the composition and metabolism of the cell envelope[13]. Similarly, a promoter consensus search strategy resulted in the identification of 30 functional target σ^R promoters of *S. coelicolor*, an ECF that is involved in the response to disulphide stress[29]. Transcriptional profiling microarray analysis proved successful in identifying many target promoters of two stress response ECF factors (σ^E and σ^H) of *M. tuberculosis*[22, 23]. It is therefore likely that future strategies in the post-genomic era for understanding the function of ECF genes will be (a) transcriptional profiling if the inducing conditions of the particular ECF are known, (b) a computer based promoter consensus search method; however this will work well only if the promoter recognition sequence is known and is highly conserved, (c) classical genetic studies aimed at localizing genes which are switched on or off in different ECF genetic backgrounds and (d) proteome analysis of total proteins of the wild-type strain compared to the ECF knockout mutant. Only a combination of these strategies will lead to the complete identification of a sigma regulon.

In *P. aeruginosa*, the σ^E and σ^{PvdS} ECF regulons have been recently studied resulting in the identification of many target promoters. The σ^E regulon has been investigated aided by a promoter consensus search, transcriptional profiling, proteome analysis and classical genetics[10, 11, 21], whereas the σ^{PvdS} regulon has been studied by transcriptional profiling and by classical genetics[28, 41].

2.2. The *P. aeruginosa* σ^E Regulon

The σ^E factor was initially identified as a regulator of the biosynthesis of the exopolysaccharide alginate, consequently several alginate biosynthesis

genes, including *algR, algD, algC* and *algZ* were demonstrated to be directly regulated by σ (see refs [2], [12], [14]). In addition, σ^E like many ECF members, is autoregulatory by directing transcription of its own gene *algU*[14]. The identification of these five σ^E regulated promoters revealed the following tight promoter consensus sequence [(−35) GAACTT—$N_{16/17}$—(−10) TctgA]. This consensus sequence was subjected to a computer search against the *P. aeruginosa* PAO1 genome resulting in the identification of 35 potential σ^E promoters. Of these, 10 promoters were subjected to further experimentation and confirmed to be σ^E dependent (Table 2)[10]. Obviously this number could considerably increase once the putative remaining promoters identified with the consensus search are experimentally investigated. In addition, it is likely that the consensus search did not detect all σ^E regulated promoters since some could deviate from the consensus by one or two residues as is the case with the *algR* and *osmE* promoters (see below and Table 2). Among the identified σ^E regulated genes are two genes (*lptA* and *lptB*), which encode two putative lipoproteins that might play a role in the inflammatory response in the fibrocystic lung infected with *P. aeruginosa* through Toll-like receptor mediated inflammation. In fact experiments have shown that peptides corresponding to the LptA and LptB N-termini caused IL-8 secretion by primary human macrophages[10].

The study of the σ^E regulon has also been in part investigated by proteome analysis looking at global protein expression in *P. aeruginosa*. A 2-D analysis of total proteins of *P. aeruginosa* and a *mucA* mutant derivative revealed that six proteins were up-regulated in the absence of the σ^E–anti-sigma factor MucA[21]. The inner membrane MucA protein is an anti-sigma factor which controls the activity of σ^E thus in the MucA mutant, σ^E is released and activates transcription indicating that the six up-regulated proteins are likely to be σ^E regulated. Three of the six proteins identified (AlgD, AlgA and OprF) were already known to be σ^E regulated, another one was a homologue of the *E. coli* ClpP periplasmic serine protease, one was a protein of unknown function and the last being DbsA, a periplasmic disulphide bond isomerase. These σ^E controlled genes have still to be characterized at the gene promoter level. This technique proved successful however it identified a rather small number of proteins; further optimization of this technique and the analysis of the σ^E null mutant vs the wild-type strain might add several new genes to this regulon.

A transcription profile microarray analysis of σ^E-dependent expression in a *P. aeruginosa* mucoid strain was also recently performed[11]. Most of the already known σ^E regulated genes were detected, however many new genes were also found (Table 2) considerably increasing the number of known σ^E dependent promoters making this one of the best-studied ECF factor regulons in bacteria. Among the new genes found were (a) *pfpI*, encoding for a putative protease, (b) *osmE*, encoding an osmotically inducible lipoprotein, (c) genes encoding for several membrane proteins including an ABC transporter and

Table 2. σ^E regulated promoters of *P. aeruginosa*.

P. aeruginosa promoter[a]	Target −35 and −10 sequence[b]	Identification strategy	References
oprF	GAACTT—17 bp—TCAAA	Promoter consensus search	[10]
lptA	GAACTT—17 bp—TCAAA	Promoter consensus search	[10]
asmA6	GAACTT—16 bp—TCTGA	Promoter consensus search	[10]
asmB2	GAACTT—17 bp—TCCCA	Promoter consensus search	[10]
osmC	GAACTT—17 bp—TCAAA	Promoter consensus search	[10]
asmC1	GAACTT—17 bp—TCAGA	Promoter consensus search	[10]
asmD2	GAACTT—17 bp—TCCAA	Promoter consensus search	[10]
asmD3	GAACTT—16 bp—TCCAA	Promoter consensus search	[10]
slyB	GAACTT—16 bp—TCCTA	Promoter consensus search	[10]
lptB	GAACTT—16 bp—TCCTA	Promoter consensus search	[10]
algU	GAACTT—16 bp—TCTAT	Genetic and molecular studies	[24]
algD	GAACTT—17 bp—TCCTA	Genetic and molecular studies	[24]
algR	GCACTT—17 bp—TCTCA	Genetic and molecular studies	[24]
rpoH	GAACTT—16 bp—TCAGA	Genetic and molecular studies	[24]
dsbA	Currently unknown	Proteome analysis	[21]
algA	Currently unknown	Proteome analysis	[21]
osmE	GAACTC—17 bp—TCCAA	Transcriptional profiling	[11]
PA5182	Currently unknown	Transcriptional profiling	[11]
PA3890	Currently unknown	Transcriptional profiling	[11]
PA2777	Currently unknown	Transcriptional profiling	[11]
PA3041	Currently unknown	Transcriptional profiling	[11]
PA2148	Currently unknown	Transcriptional profiling	[11]
pfpI	Currently unknown	Transcriptional profiling	[11]
galE	Currently unknown	Transcriptional profiling	[11]
nirN	Currently unknown	Transcriptional profiling	[11]
nirJ	Currently unknown	Transcriptional profiling	[11]
PA2007	Currently unknown	Transcriptional profiling	[11]
bkdB	Currently unknown	Transcriptional profiling	[11]
σ^E promoter consensus[c]			
P. aeruginosa	GAACTT—16/17 bp—TCcaA		[10]
E. coli	gAActT—16 bp—TCTgA		[8]
M. tuberculosis	GGGAAT—17—cGTTg		[23]
S. coelicolor	GAACTT—16—CTCTC		[19]

This table was adapted in part from Firoved *et al.*, 2002.

[a]Listed here are genes controlled by the target σ^E regulated promoters. PAXXXX refers to a gene with a homology proposed function (see annotated *P. aeruginosa* genome at www.pseudomonas.com). PA51682, PA2777, PA3041 and PA2148 encode hypothetical membrane proteins; PA3890, probable permease of ABC transporter; PA2007, probable maleylacetoacetate.

[b]"Currently unknown" refers to unmapped gene promoters, all others refer to experimentally mapped −35 and −10 promoter regions.

[c]σ^E target consensus derived from different bacterial species.

(d) several genes encoding for metabolic proteins (see Table 2 for further details). From this and other studies it appears that σ^E regulates not only alginate production but also genes encoding proteins involved in adhesion, in drug resistance mechanisms (e.g., efflux pumps), having protease activity and enzymes having metabolic potential. One of the newly identified genes using this technique was further investigated and its promoter sequence mapped. The *osmE* gene was shown to have a very close σ^E promoter consensus sequence (Table 2) and future mapping of the other gene promoters will most probably also reveal −35 and −10 sequences close to the consensus.

Table 2 is a compilation of all the characterized *P. aeruginosa* σ^E regulated promoters identified thus far using the various described approaches and it can be seen that a very stringent consensus is evident. Interestingly, some σ^E homologues of other bacteria share striking similarity among the cognate promoters that they recognize[26]; the −35 sequence is well conserved whereas the −10 region has a lower degree of resemblance (Table 2 also contains the consensus sequence of σ^E promoters of other bacteria). This suggests that σ^E ECF factors of different species have rather similar promoter specificity; in fact the *P. aeuriginosa* σ^E is capable of recognizing the *rpoH*P3 *E. coli* σ^E promoter[14].

2.3. The *P. aeruginosa* σ^{PvdS} Regulon

The σ^{PvdS} ECF factor is essential for the expression under iron limiting conditions of all the pyoverdine biosynthesis genes examined thus far including *pvdA, pvdD, pvdE* and *pvdF*; in addition it regulates the expression *ptxR*, a gene which encodes for PtxR, a LysR type regulator which controls the *pvc* genes that direct synthesis of the chromophore component of pyoverdine[35, 39, 41]. σ^{PvdS} is also required for the synthesis of exotoxin A and of the PrpL extracellular proteinase[17]. Purified σ^{PvdS} has been shown to bind to several *pvd* promoters where a DNA sequence at the −35 region designated as "IS box" is important for proper promoter functioning through specific recognition by σ^{PvdS} thus enabling initiation of transcription[40, 41]. All promoters that are known to be regulated by σ^{PvdS} contain IS box sequences at the −35 promoter region composed of G/C G/C TAAAT T/A C/G (Table 3). This is in accordance with other ECF members which tend to have a highly conserved −35 recognition motif; the *pvdE* pyoverdine biosynthesis gene promoter however has the IS box centred at the −19 position, this could be due to the divergent nature of its promoter as it overlaps and interacts with the *pvdF* promoter[41].

The study of the σ^{PvdS} regulon in *P. aeruginosa* has also been recently studied by microarray transcriptional profiling demonstrating that in a *pvdS* knockout mutant, expression of 26 loci was reduced under iron limiting conditions[28]. Among the 26 loci, the known σ^{PvdS} regulated genes were present and new pyoverdine biosynthesis genes were identified, such as a gene located

Table 3. σ^{PvdS} regulated promoters of *P. aeruginosa*.

P. aeruginosa promoter[a]	Target −35 and −10 sequence[b]	Identification strategy	References
pvdA	TAAAT—16 bp—CGT	Genetic and molecular studies	[41]
pvdD	TAAAT	Genetic and molecular studies	[41]
pvdE	TAAAT—16 bp—CGT	Genetic and molecular studies	[41]
pvdF	TAAAT—16 bp—CGT	Genetic and molecular studies	[41]
pvdY	TAAAT—16 bp—CGT	Transcriptional profiling	[28]
pvdL	TAAAT—16 bp—CGT	Transcriptional profiling	[28]
PA2383-2384	na[c]	Transcriptional profiling	[28]
PA2411-2412	TAATT—16 bp—CGT	Transcriptional profiling	[28]
PA2413	TAAAT—16 bp—CGT	Transcriptional profiling	[28]
PA2451	CAAAT—16 bp—CGT	Transcriptional profiling	[28]
PA2393-2395	TAAAT—16 bp—CGT	Transcriptional profiling	[28]
fpvA	TAAA	Transcriptional profiling	[28]
pvdJ-pvdI	TAATT—16 bp—CGT	Transcriptional profiling	[28]
PA2403-2410	na	Transcriptional profiling	[28]
PA2389-2391	TAAAT—16 bp—CGT	Transcriptional profiling	[28]
PA2392	TAAAT—16 bp—CGT	Transcriptional profiling	[28]
PA2531	TAAAG—16 bp—CGT	Transcriptional profiling	[28]
PA5150	GAAAG—16 bp—CGT	Transcriptional profiling	[28]
toxR	na	Transcriptional profiling	[28]
PA1134	TAAAC—16 bp—CGT	Transcriptional profiling	[28]
prpL	TAAAA—16 bp—CGT	Transcriptional profiling	[28]
PA0346	TGAAT—16 bp—CGT	Transcriptional profiling	[28]
PA0818	TGAAT—16 bp—CGT	Transcriptional profiling	[28]
PA4833	TAAAA—16 bp—CGT	Transcriptional profiling	[28]
PA1003	TAAAT—16 bp—CGT	Transcriptional profiling	[28]
toxA	TAAAT	Transcriptional profiling	[28]

This table was adapted in part from Ochsner *et al.*, 2002.

[a]Listed here are genes controlled by the target σ^{PvdS} regulated promoters. PAXXXX refers to a gene with a homology proposed function (see annotated *P. aeruginosa* genome at www.pseudomonas.com). PA2383 and PA1003 encode for a LysR-type regulator; PA2411 encodes for a probable thioesterase; PA2413 and PA2531 encode a probable aminotransferase; PA2393-2395 encode for a dipeptidase, for an aminotransferase and for a reductase respectively; PA2407 encodes for a probable adhesin; PA2408 and PA2409 encode for ABC transporter proteins; PA2390 encodes for an ABC transporter protein; PA2391 encodes for an outer membrane protein; PA5150 encodes for a probable short-chain dehydrogenase; PA4833 encodes for a protein similar to haemolysin Hly-III family; PA2384, PA2412, PA2541, PA2403, PA2404, PA2405, PA2406, PA2410, PA2389, PA2392, PA1134, PA0346 and PA0818 encode for hypothetical, unclassified and unknown proteins.

[b]All promoter −35 and −10 target sequences listed with σ^{PvdS} regulated genetic loci identified by transcriptional profiling are putative as the promoters have not been experimentally mapped. The *pvdE* gene has its IS box "TAAAT" centered around the −19 position (see text for details).

[c]na, not applicable.

upstream *pvdA* encoding for a putative 84 kDa acylase, and other genes encoding a putative dipeptidase, an aminotransferase and an iron(iii)-reductase (Table 3). Other genes were also observed to be under σ^{PvdS} regulation, including genes encoding a basic 23 kDa membrane protein with high identity to the

haemolysin protein family, a LysR-type transcriptional regulator located next to the *phnAB* phenazine biosynthesis operon and two genes encoding for extracellular proteins of unknown function (Table 3). Almost all of the newly identified σ^{PvdS} regulated genes contained an IS box sequence in their putative promoter region (Table 3) confirming the strict requirement of this sequence for the initiation of transcription by core polymerase with σ^{PvdS} as sigma factor. The few loci which did not contain this IS box sequence are probably very indirectly regulated by σ^{PvdS} and future work will determine this.

In *P. putida*, siderophore biosynthesis is regulated by σ^{PfrI}, a functional orthologue of σ^{PvdS}; in fact *P. aeruginosa pvd* promoters are also regulated in *P. putida* by σ^{PfrI18} and the two ECF sigma factors share 85% amino acid identity. Unlike for σ^{PvdS}, the σ^{PfrI} regulon of *P. putida* has not been studied; only one promoter has been mapped (not surprisingly, it contains an IS box centred at the −35 region) regulating the *ppsD* gene which encodes a siderophore peptide synthetase[9] (the *P. aeruginosa pvdD* homologue). Considering that the two sigma factors are functional orthologues and that the *P. putida* complete genome sequence is now available[27], the IS box consensus sequence and its derivatives having permutations at one bp position, were searched against the *P. putida* genome sequence (this study; Table 4). The results of this genome bioinformatic consensus sequence search are shown in Table 4; the most common conserved σ^{PvdS} IS box observed from the studies of *P. aeruginosa* (i.e., TAAAT—16 bp—CGT) gave 15 perfect matches in the *P. putida* genome, 10 of which are in potential gene/operon promoter regions (data not shown). The IS box sequences containing substitutions, which were observed in some putative IS boxes of σ^{PvdS} controlled genes identified by transcriptional profiling (Table 3), gave many more hits in the *P. putida* genome making their computer analysis and potential experimental evaluation more difficult. The prediction

Table 4. Number of putative σ^{PfrI} target sites in *P. putida*.

IS box consensus sequence[a]	Number of matches in *P. putida* genome[b]
TAAAT—16 bp—CGT	15
TAAAN—16 bp—CGT	186
T A/G AAT—16 bp—CGT	55
T/C/G AAAT—16 bp—CGT	112

[a]These are all possible promoter consensus target sequences for σ^{PfrI} which have been derived from all possible combinations of the *P. aeruginosa* σ^{PvdS} target sequences (Table 3; see text for details).
[b]This number refers to the number of sequences which match the consensus displayed on the same row (see text for details).

of these IS boxes is a starting point in the deciphering of the *P. putida* σ^{PfrI} regulon which might prove important in understanding the differences between *P. aeruginosa* and *P. putida* adaptation to iron limitation as they are competitive colonizers of different habitats.

2.4. Other *Pseudomonas* σ^{ECF} Regulons

In *P. fluorescens*, σ^{PrtI} was shown to regulate the *aprX* gene encoding a secreted protease[6], the *aprX* promoter was localized and the putative −35 and −10 regions are given in Table 1. This is the only gene known so far to be regulated by σ^{PrtI} and more work is necessary in order to establish if more genes are regulated by this ECF factor. Similarly, σ^{SigX} of *P. aeruginosa* and *P. fluorescens* has been shown to regulate one gene, the major outer-membrane protein (OprF) encoding gene *oprF*[5]. The mapping of the *oprF* promoter in both species revealed that two transcriptional initiation points were present, one initiated via σ^{70} and the other by σ^{SigX}. Interestingly, the σ^{SigX}-dependent site showed a −35 promoter region of GAAGTT, very similar to the *P. aeruginosa* σ^{E} −35 consensus sequence (see Tables 1 and 2), whereas the −10 region showed no conservation with other known −10 regions of *Pseudomonas* ECF members highlighting once again that this region is not normally conserved. The *oprF* gene in fact has also been shown to be regulated by σ^{E} (see above)[10], thus this observed homology in the −35 promoter regions of σ^{SigX} and σ^{E} could reveal that two ECFs regulate the same gene promoter maybe under different environmental conditions. Most of the *Pseudomonas* ECF factors have still to be studied as their function and target genes remain unknown. It is possible that many (14/19) are involved in iron acquisition and/or iron regulation of colonization and virulence genes[25, 39].

3. CONCLUDING REMARKS

In *Pseudomonas*, like in many other bacteria, many ECF factors have been identified based on sequence homology, however only relatively few have been characterized. It will be a challenge for scientists in the future to perform experimental studies and determine the stimuli resulting in activation of the sigma factors and to identify the corresponding regulons. ECF factors are found in a diverse spectrum of bacteria and control a wide range of functions such as response to heat, osmotic, oxidative stresses, virulence, motility, transport of metal ions and synthesis of alginate and carotenoids. The number of ECF factors within a bacterial species is correlated with metabolic and developmental complexity as well as with genome size. Inspection of phylogenetic relatedness

among various ECF factors reveals several sub-clusters with related functions[13, 25, 26, 39], for example (a) an iron transport cluster which is especially numerous in *Pseudomonas*; (b) a disulphide stress cluster evident from factors of *Mycobacterium* and *Streptomyces*; and (c) a cluster of homologues of σ^E which may regulate periplasmic and heat responses. Both in *P. putida* and *P. aeruginosa* 14/19 of the ECFs present are involved in iron assimilation highlighting the highly evolved response to the iron availability in a specific environment[25, 39].

Studies in *Pseudomonas* have concentrated on the σ^E and σ^{PvdS} regulons; two factors controlling a rather large number of loci, each factor requiring a stringent −35 and −10 promoter target sequence (Tables 1, 2 and 3). It is possible that several of the uncharacterized *P. putida* and *P. aeruginosa* ECF factors are involved in the regulation of fewer loci and some as few as one[16, 25, 39]. It is predicted that as many as 10 ECF factors in these two *Pseudomonas* species could each be regulating one outer-membrane receptor gene involved in iron transport which is localized in the chromosome adjacent to the ECF factor and its cognate anti-sigma factor genes. These 10 loci consisting of an ECF factor/anti-sigma factor/outer-membrane receptor genes are likely to constitute a signal transduction system similar to the *Pseudomonas* Fpv and Pup systems, which are induced by the presence of cognate iron carriers[16, 17, 25, 39].

Future studies on *Pseudomonas* ECF factors as well as in other genera like *Bacillus, Streptomyces* and *Mycobacterium* will reveal how a bacterium copes with having many similar sigma factors. A comparison of different ECF factors reveals that in the promoter target sequence, the −35 sequence and spacing but not the sequence between the −35 and −10 regions share some conservation. This is especially true for sigma factors which belong to the same cluster as for example is the case with σ^E homologues of different species (Table 2). The −10 regions on the other hand show no conserved sequences which is reflected by the low degree of resemblance in region 2.4 in the members of the ECF sub-family. This diversity probably accounts for the coexistence of many members of this class of sigma factors in the same species. It has been observed however that in bacteria with a large number of ECF factors, some regulon overlap does occur. In *B. subtilis* for example, there is some overlap with the σ^W and σ^X regulons as is the case in *M. tuberculosis* between σ^H and σ^{E13}; these overalps are believed to be at least in part due to overlapping recognition properties in the −10 consensus element. A similar situation of overlap could occur with the *oprF* promoter in *P. aeruginosa* which appears to be regulated by both σ^E and σ^{SigX} (see above). Extensive studies on ECF factor target genes and promoters within a species will reveal the extent of regulon overlap. In addition to promoter stringency, specific transcription by a specific ECF factor could also be modulated by the availability of the sigma factor since many are "released" or "activated" by another protein(s) following an environmental signal.

Novel technologies such as high-throughput sequencing, transcriptional profiling by DNA microarrays, proteomics and computer data analysis now allow a rather fast and complete analysis of a specific sigma factor regulon. Computer aided definition of ECF target gene promoters has proved particularly successful, examples being the σ^E regulon of *P. aeruginosa*[10] as well as for several other regulons of *B. subtilis* and *S. coelicolor*[13]. Target promoters controlled by ECF factors usually benefit from a high degree of conservation as the σ factors are often controlled by a ligand-responsive σ–anti-σ interaction rather than the requirement of a positive activator. In addition, the presence of several ECF factors in the same bacterium is likely to result in a high level of promoter conservation within each class of elements despite possible similarities in the −35 recognition regions. This and the other sophisticated technologies will quickly and considerably advance our knowledge in this field in *Pseudomonas* and other bacterial species having a large set of ECF factors.

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CATABOLITE REPRESSION AND PHYSIOLOGICAL CONTROL

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1. INTRODUCTION

When confronted with a mixture of potential carbon sources at sufficiently high concentrations, many bacterial species often assimilate the different compounds in an ordered fashion, so that expression of the pathway for the assimilation of the non-preferred substrate remains inhibited until the preferred one is consumed. Pseudomonads are no exception to this. This phenomenon is, at least at first sight, similar to the "catabolite repression" control initially described in *Escherichia coli* for the hierarchical assimilation of sugars[39]. Catabolite repression has been studied mainly in *E. coli* and *Bacillus subtilis*, where it was found to be the consequence of a complex global regulatory response. In *E. coli*, the transport of glucose by the phosphotransferase sugar transport system (PTS) is coupled to its phosphorylation, a process that activates mechanisms to impede the import of other sugars[32, 55]. This process, named inducer exclusion, prevents expression of the catabolic pathways for other alternative sugars by lowering the intracellular concentration of the corresponding inducer. When glucose is consumed the levels of cAMP increase to levels that allow its binding to the cAMP receptor protein (CRP).

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The cAMP–CRP protein can then bind to a large number of promoters, where it acts as a transcriptional activator or repressor[32, 55]. Many of the activated promoters correspond to catabolic pathways for other alternative sugars. A protein called Cra also regulates the metabolism of carbohydrates in *E. coli*. This transcriptional regulator represses expression of genes for sugar catabolism and activates genes for gluconeogenesis depending on the levels of catabolites such as fructose-1-phosphate or fructose-1,6-bisphosphate[56]. In *B. subtilis*, catabolite repression occurs mainly by the action of two proteins, HPr and CcpA. The phosphorylation state of HPr determines whether it controls the activity of some regulators of catabolic operons, or acts as a corepressor of CcpA. In the presence of fructose 1,6-bisphosphate, the HPr–CcpA complex binds to DNA to regulate the expression of many catabolic promoters[64]. In spite of some apparent similarities, the precise mechanisms leading to carbon catabolite repression are significantly different in the two bacterial species. In the case of Pseudomonads, the mechanisms responsible for this global control are not well understood. As explained below, available evidence shows that this regulatory process is mechanistically different from those of *E. coli* or *B. subtilis*. In Pseudomonads, the process has been analyzed for the catabolism of some sugars, amino acids, hydrocarbons and aromatic compounds. Rather than providing a comprehensive list of the many catabolic pathways controlled by catabolite repression, the aim of this chapter is to examine how this phenomenon takes place by considering examples that have been investigated in more depth. The evidence gathered to date suggests that catabolite repression is just but one of several kinds of regulatory responses to environmental and physiological signals that modulate the expression of catabolic pathways in Pseudomonads[8]. As described below, factors other than the carbon source itself, such as availability of alternative sigma factors[67], of global regulatory proteins like IHF[74], or components of the electron transport chain that serve to monitor the physiological status or oxygen availability[21, 22, 48], can have a great impact on the induction of catabolic pathways.

2. COMPOUNDS REPRESSING THE INDUCTION OF CATABOLIC PATHWAYS

Pseudomonads have a great metabolic versatility, being able to assimilate a large variety of organic compounds[62]. Although glucose is the preferred carbon source for many bacteria, Pseudomonads preferentially metabolize many organic acids or amino acids over sugars[16]. For example, in the presence of succinate and glucose, the activity of enzymes of the central pathway of hexose catabolism such as glucose-6-phosphate dehydrogenase or 2-keto-3-deoxy-6-phosphogluconate aldolase are repressed until succinate is consumed[16].

Glucose, however, has a clear repressing effect on expression of some genes, for example on the *Pseudomonas aeruginosa* regulons for mannitol or histidine utilization, or on the amidase genes (ref. [16] and references therein). However, glucose does not play the same central role as it does in *E. coli* or *B. subtilis*. Unlike in these two microorganisms, the transport of glucose in *Pseudomonas* is not carried out by a PTS transport system. In both *Pseudomonas aeruginosa* and *Pseudomonas putida*, catabolism of glucose requires its oxidation in the periplasm to gluconate, followed by the induction of a specific energy-dependent gluconate uptake system[57]. Once internalized, gluconate is phosphorylated and enters central metabolism as 6-P-gluconate. However, when *P. aeruginosa* grows anaerobically, or under low oxygen concentrations, glucose can also be transported by an inducible active glucose-specific transport system[54]. The low preference of Pseudomonads for glucose may be the consequence of the wide range of substrates that members of this bacterial genus can use as carbon sources, which might have favoured an evolution towards a low specialization. Pseudomonads are found in terrestrial, freshwater and marine environments, as well as in association with plants and animals[60]. Metabolic versatility is probably important for such ecological diversity. However, many ubiquitous compounds that are frequently metabolized by *Pseudomonas* sp. are not the preferred growth substrates. Hydrocarbons and aromatic compounds are a clear example. These products are generated by plants and many organisms, besides being present in fossil fuels, and are therefore rather widespread in nature. Nevertheless, expression of the corresponding catabolic pathways is normally inhibited in the presence of many organic acids, glucose or amino acids[23, 30, 40, 42, 43, 44, 61, 69, 73, 79].

Not all organic acids can mediate a catabolite repression effect and, even more intriguingly, some of them have a clear effect in some cases, but not in other ones. For example, citrate allows a good expression of the *P. putida* GPo1 OCT plasmid alkane degradation pathway[61, 79], but has a repressive effect on the *P. putida* H phenol degradation pathway[43] and on the *P. putida* CA-3 styrene pathway[44]. This may be ascribed to particular differences in the bacterial strains analysed, or in the experimental conditions. The repressing effect of organic acids such as succinate, lactate or pyruvate, is more evident when cells are growing exponentially, and fades away when cells approach the stationary phase, even when the organic acid is still present in the medium. Overall, available data suggest that the repression of catabolic pathways frequently results from the integration of several signals, rather than just from the presence of a preferred compound in the medium, an idea that will become more apparent later on in this chapter. Finally, it is important to note that carbon catabolite repression is observed only when the preferred carbon source is present at high concentrations. This is a long-known and generally occurring phenomenon (ref. [36] and references therein) that, in the case of

Pseudomonads, has been studied in detail for the hydrocarbon degradation pathways specified in the *P. putida* plasmids pWW0 (toluene/xylene oxidation) and OCT (alkane oxidation). In these two cases, growth of cells in continuous cultures has shown that the presence of succinate at concentrations that allow non-limited growth (maximal growth rates) generates a strong catabolite repression effect, while succinate concentrations that lead to carbon-limitation conditions do not generate the repressing effect[21, 23, 24].

3. FACTORS INVOLVED: COMPONENTS MEDIATING THE REPRESSION EFFECT

Modulation of the induction of catabolic pathways by metabolic and/or physiological cues requires the detection and integration of signals, followed by the generation of a final output response that should be transmitted to a proper receptor, for example, the specific regulation system of a catabolic promoter. In Pseudomonads, knowledge on the components of these signal-transmission pathways is far from complete with only a few factors having been genetically identified. Interestingly, each pathway seems to respond just to a subset of these factors, being insensitive to others. Similarly, some factors are important only under certain metabolic conditions, while being irrelevant in other situations or for other repressing effects. It appears that the possible participation of a protein similar to the *E. coli* CRP can be discarded. A protein showing high similarity to *E. coli* CRP is present in *P. aeruginosa* (83% similarity), *P. fluorescens* (82% similarity) and *P. putida* (80% similarity). In *P. aeruginosa*, this protein has been called Vfr, and its function has been analyzed with some detail[1, 65]. Evidence gathered to date indicates that Vfr is a global regulator of gene expression. However, it is not involved in catabolite repression, but in regulation of the quorum sensing response[1, 65]. Vfr can complement a mutation in the *E. coli crp* gene, where it requires cAMP to bind DNA. The levels of cAMP in Pseudomonads do not seem to vary in response to the nature or concentration of different carbon sources, or along the growth phase[49, 59]. Therefore, cAMP may not be the only ligand that binds to and activates Vfr.

The factors identified as having a clear effect in modulation of the induction of catabolic pathways in Pseudomonads are described below.

3.1. Crc

The first protein described participating in catabolite repression in *Pseudomonas* was Crc (*c*atabolite *r*epression *c*ontrol). This protein is involved in the repression of a number of genes implicated in the metabolism of some

sugars, amino acids and nitrogenated compounds both in *P. aeruginosa*[16, 37, 78], and in *P. putida*[28, 29]. However, not all genes regulated by catabolite repression are influenced by Crc. For example, the histidine utilization regulon is still subject to a strong catabolite repression effect by succinate in Crc-deficient strains[16, 28, 78].

Despite several efforts, the mechanism by which Crc regulates gene expression has not yet been elucidated. Crc has sequence similarity (25–38% identity) to several deoxyribonucleases and DNA repair enzymes. However, it does not appear to have endo- or exonuclease activity or to bind DNA[37]. The phenotype of *crc* null mutants indicates that this protein acts to repress gene expression in the presence of a preferred carbon source, rather than activate gene expression when the preferred carbon source is absent[37]. Induction of the *P. putida bkd* operon, which encodes the branched-chain keto-acid dehydrogenase, is down-modulated by glucose or by growth of cells in a rich medium. Expression of this operon requires the BkdR transcriptional activator and branched-chained amino acids. When cells are grown in rich medium, inactivation of the *crc* gene leads to elevated levels of BkdR, although the amount of *bkdR* mRNA remains unchanged. This was interpreted as indicating that Crc has a direct or indirect post-transcriptional effect on *bkdR* expression[28, 29]. The Crc protein also represses expression of the *P. putida* GPo1 OCT plasmid alkane degradation pathway (the *alk* pathway)[80]. This pathway is controlled by the transcriptional regulator AlkS, which activates expression of its own gene from a promoter named *PalkS2* and also that of most of the structural genes of the pathway from promoter *PalkB*[5] (see Figure 1A). When cells are grown in a rich medium containing alkanes, induction of the *PalkS2* and *PalkB* promoters by alkanes is strongly repressed during the exponential phase of growth[5, 79]. Repression abruptly disappears at the onset of the stationary phase. Inactivation of the *crc* gene leads to elevated expression of the *PalkS2* and *PalkB* promoters during the exponential growth. However, repression relief is only partial, suggesting that other factors besides Crc participate in the modulation of this pathway[80] (see below). The repressing effect of the rich medium is totally abolished if the AlkS protein is overexpressed from a heterologous promoter. Since AlkS is an unstable protein present in limiting amounts, it has been proposed that Crc acts to decrease the levels of the AlkS regulator, thereby controlling expression of the complete pathway[80]. Considering that AlkS activates its own synthesis, Crc could modulate expression of *alkS* at the transcriptional or at a post-transcriptional level, because both strategies would lead to the same final effect, that is, to reduce the levels of AlkS. Taking into account the example of BkdR described above, a regulator that does not activate its own synthesis, it seems likely that Crc regulates expression of *alkS* post-transcriptionally. Crc has little effect on the stability of the transcripts arising from the *PalkS2* and *PalkB* promoters[80], so it probably acts by

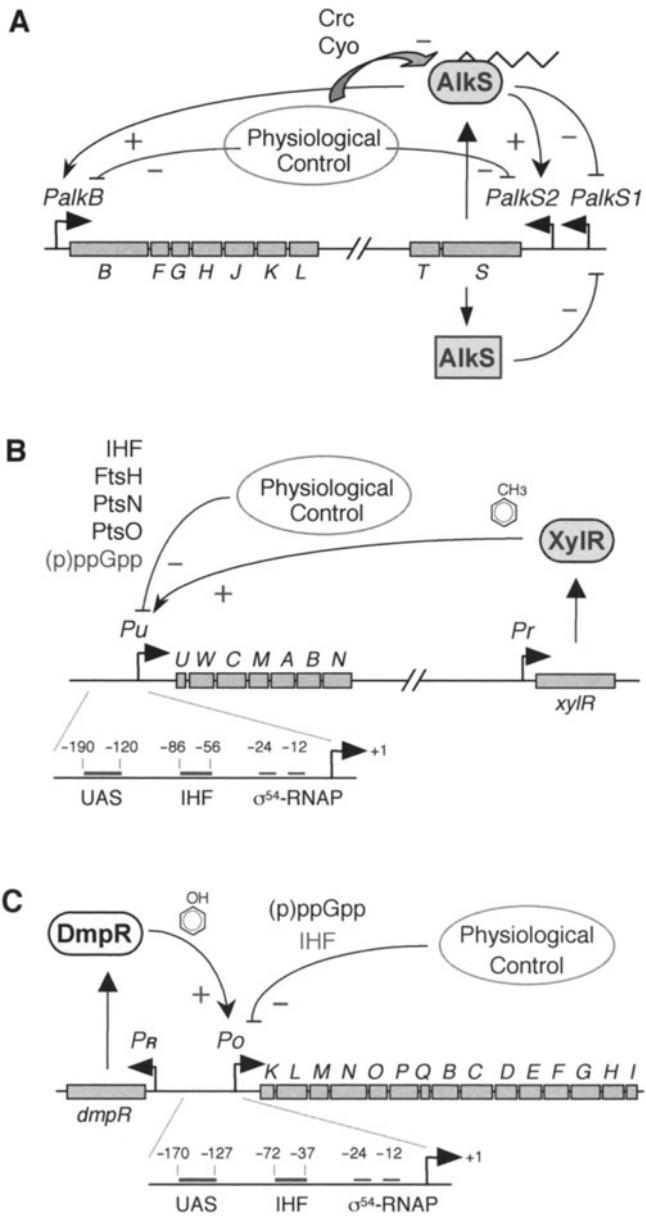

Figure 1. Organization and regulation of the *alk*, TOL and *dmp* catabolic pathways. (A) The *P. putida* OCT plasmid alkane degradation pathway. The genes are grouped into two clusters, *alkBFGHJKL* and *alkST*, both of which are regulated by the AlkS protein. In the absence of alkanes *alkS* is expressed at low levels from promoter *PalkS1*, which is recognized by σ^s-RNA

inhibiting translation of the *alkS* mRNA. No obvious common sequence pattern is evident upstream of the genes regulated by Crc. Furthermore, the sequences upstream from the AlkS binding site at the *PalkB* promoter can be eliminated with no influence on the repression effect observed in rich medium. Therefore, the current data suggest that Crc may recognize an RNA secondary structure, an idea consistent with the sequence similarity of Crc to DNA repair enzymes. It can be speculated that the role of Crc could be to recognize and bind this structure, stabilizing it and inhibiting translation, for example, by hindering the access of ribosomes to the Shine–Dalgarno sequence. However, there is no experimental evidence at present to support or refute this idea.

Interestingly, Crc seems to participate in the transduction of nutritional signals that trigger biofilm development. *P. aeruginosa crc* mutants are defective in type IV pilus-mediated twitching motility, at least in part because of a decreased transcription of *pilA*, which codes for pilin. This in turn impairs adhesion and biofilm formation[45]. It is interesting to note that biofilm formation is inhibited by the presence of glucose in *E. coli*[33] and in *B. subtilis*[63]. In both cases, the proteins that transmit the inhibitory signal are also involved in catabolite repression control. Therefore, biofilm formation in these bacteria is stimulated by non-optimal growth conditions. Environmental and nutritional cues are also important for biofilm development in *P. aeruginosa*, and Crc seems to integrate or transmit these signals. Taken together, the available data suggest that Crc could be a component of a signal transduction pathway modulating carbon metabolism. However, no other components of this hypothetical pathway are known. Several *P. aeruginosa* pseudorevertants containing

Figure 1. Continued
polymerase (RNAP). AlkS negatively modulates this promoter to keep a low and constant expression. When alkanes are available, AlkS activates transcription from the *PalkB* and *PalkS2* promoters, which leads to a self-amplification of *alkS* expression. Activation of these two promoters is negatively modulated by a dominant global control when cells grow in the presence of alternative carbon sources, a process mediated by Crc and Cyo (see text for details). Inhibition of *PalkS2* leads to a decrease in AlkS levels, an instable protein present in limiting amounts. (Modified from refs [5], [79]). (B) The *P. putida* TOL plasmid pWW0 *upper* pathway for assimilation of toluene. The genes required to oxidate toluene to benzoate are expressed from the *Pu* promoter. Transcription from *Pu* requires σ^{54}-RNAP, the IHF protein, an effector such as toluene, and the transcriptional regulator XylR, which binds to the UAS sequence (see the enlarged region). XylR activation of *Pu* is modulated by the levels of IHF, the activity of the FtsH, PtsN and PtsO proteins and, to a lower extent, by the levels of (p)ppGpp (see text for details). (C) The (methyl)phenol degradation pathway encoded in the *P. putida* pVI150 plasmid. All the structural genes are expressed from the *Po* promoter, whose expression requires σ^{54}-RNAP, IHF, (methyl)phenol and the transcriptional regulator DmpR, which binds to the UAS region (see the enlarged area). DmpR activation of *Po* is modulated primarily by the levels of (p)ppGpp, while IHF has a lesser role (see text for details).

suppressors for an inactivated *crc* allele have been isolated[17]. These mutations do not restore *crc* expression and map outside of *crc*, at loci that have not been identified. The possibility exists that they may affect elements of the hypothetical signal cascade acting either "upstream" or "downstream" of Crc.

3.2. The Cytochrome *o* Ubiquinol Oxidase

A random transposon-mutagenesis strategy has identified a connection between the activity of the electron transport chain and the repression of catabolic pathways by preferred carbon sources and other physiological and environmental signals. The aim was to isolate insertion mutants showing a reduced repression effect of alternative carbon sources, and was independently applied to the phenol degradation pathway encoded in the *P. putida* H pPGH1 plasmid[48], and to the *P. putida* GPo1 OCT plasmid alkane degradation pathway[22]. Several mutants were found in which the repression induced by succinate (phenol pathway), or that induced by succinate and by rich medium (alkane pathway), was partially relieved. In both cases, most of the mutants mapped to the *cyoABCDE* cluster. These genes encode the different subunits of the cytochrome *o* ubiquinol oxidase, one of the terminal oxidases of the electron transport chain. Most bacteria contain branched respiratory chains organized in modules[53] (see Figure 2). The first module gathers several substrate-specific dehydrogenases that transfer electrons (reducing equivalents) from certain substrates (NADH, succinate, etc.) to the ubiquinone pool present in the cytoplasmic membrane (the second module). These electrons are then taken from the reduced ubiquinole by a number of cytochrome oxidases that act as electron acceptors (the third module). The precise dehydrogenases and cytochrome oxidases used varies according to the nutritional and environmental conditions, a strategy that allows a flexible adaptation to changing environments[53]. In the case of *P. aeruginosa*, *P. putida* and *E. coli*, the main terminal oxidase, when cells grow under high oxygen concentrations, is the cytochrome *o* ubiquinol oxidase (a bo_3-type oxidase). Under conditions of low oxygen tension, this oxidase is substituted by a *bd*-type oxidase that has a higher affinity for oxygen[18, 19, 66]. The finding that inactivation of the cytochrome *o* ubiquinol oxidase reduces the repression of both the phenol and alkane catabolic pathways by the presence of preferred carbon sources is strong evidence supporting the idea that the electron transport chain is used to gather and transmit signals as to the physiological status of the cell. The signal could be the flow of electrons through the electron transport chain, the redox state of the cell (the ratio of reduced to oxidized ubiquinones), or the amounts of the cytochrome *o* ubiquinol oxidase, the levels of which are known to depend on factors such as the oxygen tension or the carbon source being used[18, 21, 66] (see Figure 2). It could be argued that the effect observed is indirect, for example, due to

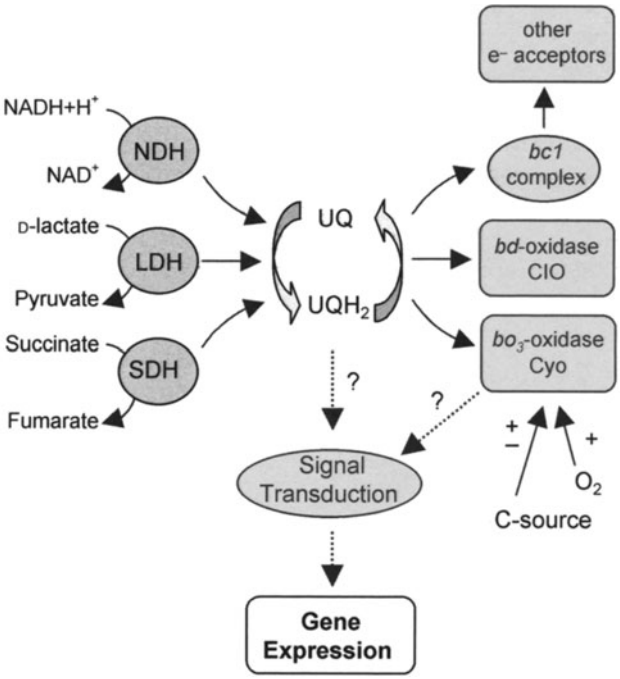

Figure 2. The respiratory chain and its connection to physiological control of gene expression. The scheme represents the three modules of the electron transport chain (dehydrogenases that transfer reducing equivalents to the ubiquinones, the ubiquinone/ubiquinol pool and the cytochromes that take the reducing equivalents from ubiquinol). The expression of the cytochrome *o* ubiquinol oxidase (Cyo) varies according to the O_2 levels and the carbon source being used. The levels of Cyo, or perhaps its effect on the ratio of ubiquinone/ubiquinol, can modulate the expression of certain catabolic pathways by means of mechanisms that remain poorly understood. NDH, NADH dehydrogenase; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase; UQ, ubiquinone; UQH_2, ubiquinol; CIO, cyanide-insensitive terminal oxidase (a *bd*-type oxidase present in *Pseudomonas*)[19].

a reduced growth rate of mutants lacking the cytochrome *o* ubiquinol oxidase. Although the absence of this oxidase is compensated by other terminal oxidases, these mutants do grow at a slightly lower rate. However, several lines of evidence indicate that growth rate *per se* cannot explain catabolic repression at the phenol and alkane degradation pathways. On the one hand, mutation of many genes could cause a reduced growth rate or an indirect effect on the expression of these two degradation pathways; however, the mutations obtained in independent screenings repeatedly mapped at the *cyoABCDE* cluster. On the other hand, growth of cells in a defined medium at low growth rates by limiting the concentration of succinate relieves the repression imposed by

this organic acid on induction of the alkane degradation pathway. However, if the same low growth rate is obtained by limiting the availability of nitrogen, providing succinate in excess, repression is not relieved[21]. Similarly, decreasing growth rate in rich medium by decreasing the temperature of the culture does not affect repression[79].

Monitoring the activity or the composition of the electron transport chain to gather information on the physiological status of the cell seems as a good strategy, and is in fact used to regulate the activity of certain genes in other cases. For example, in *Rhodobacter sphaeroides* the information obtained at two points of the electron transport chain is used to regulate the expression of photosynthesis genes in response to O_2 and other signals[46]. First, a signal generated at the cbb_3 branch of the electron transport chain is transduced to a two-component activation system that directly regulates gene expression. Second, the redox state of the quinone pool (the ubiquinol/ ubiquinone ratio) is also monitored by a redox-active antirepressor protein, which determines the functional state of a transcriptional repressor. Sensing the redox state of quinones is also used to regulate the transition from aerobic to anaerobic metabolism in *E. coli* by means of the Arc two-component system[27]. In this case, the oxidized form of the quinones serves as a specific signal for the ArcB sensor kinase, silencing it and impeding phosphorylation of the ArcA global transcriptional regulator. Therefore, bacterial cells can monitor the activity and/or the composition of the electron transport chain and use this information to regulate the expression of diverse genes. The precise mechanism that transforms the information on the activity or composition of the electron transport chain into a modulation of the expression of the phenol and alkane catabolic pathways is not yet clear. However, the levels of the cytochrome *o* ubiquinol oxidase appear to be important. Expression of the *P. putida cyoABCDE* genes is high under conditions that repress the alkane degradation pathway (high O_2 concentrations, or presence of succinate as alternative carbon source), but decrease significantly under conditions that relieve repression (low O_2 concentrations, or presence of citrate as alternative carbon source)[21]. In the case of the alkane degradation pathway, the final effect of the signal transmitted is the inhibition of the induction of the *PalkS2* and *PalkB* promoters by AlkS and alkanes. Inactivation of the cytochrome *o* ubiquinol oxidase leads to a 6–7-fold increase in the induction of these promoters[22]. As in the case of Crc, the final target is to down-modulate the levels of the AlkS activator and, in doing so, keeping induction of the pathway at a suboptimal level.

It is worth noting that the cytochrome *o* ubiquinol oxidase does not account for all the repression effect in all cases. Inactivation of this oxidase reduces, but does not eliminate, the repression exerted on both the alkane and phenol degradation pathways[22, 48]. In the case of the alkane pathway, simultaneous inactivation of the *crc* and *cyoB* genes releases repression in rich medium to a larger extent than the individual inactivation of either gene

alone[22]. This suggests that the two proteins form part of different signal transduction pathways. However, it is also possible that both proteins belong to the same pathway if we assume that their synergistic effect converges on the activity of one component of the pathway. Crc seems to have a very small role on the repression mediated by succinate on the induction of the alkane degradation genes[21, 80], while the cytochome *o* ubiquinol oxidase does participate in the repression exerted by this organic acid[21, 22]. The phenol pathway behaves in an analogous—though not identical—way. Inactivation of the *cyo* genes partially relieves the repression induced by succinate, but has no effect on the repression induced by lactate[48]. The effect of rich medium on this pathway has not been reported.

The use of continuous cultures has provided clear evidence supporting that the cytochrome *o* ubiquinol oxidase can gather information not only on the carbon source being used, but on other environmental signals as well. As explained above, when cells grow in the presence of an excess of succinate as carbon source, there is a clear repressive effect that hinders induction of the alkane degradation pathway by alkanes. However, if the concentration of succinate is reduced to a level that limits growth (carbon limitation conditions), repression decreases significantly. If under these carbon-limitation conditions oxygen concentration is suddenly reduced from 100% saturation to 40% saturation, an additional 4–5-fold decrease in repression is observed[21]. Since under the carbon-limitation conditions the repressing effect of succinate has already been relieved, the effect of oxygen shows that expression of the alkane degradation pathway is modulated not only by the presence of a preferred carbon source, but by other environmental cues such as oxygen availability. If the strain used has an inactivated allele of the *cyoB* gene, induction by alkanes is equally efficient under both high and low oxygen concentrations. This evidence, together with the fact that the levels of the cytochrome *o* ubiquinol oxidase fluctuate depending on the O_2 concentration and on the carbon source being used[21], suggests that this oxidase can integrate several disparate signals, all of which finally affect the induction levels of the alkane degradation pathway. The effect of the carbon source can be defined as true carbon catabolite repression, but the influence of other signals such as O_2 concentration cannot. Therefore, carbon catabolite repression is just one of the several factors that modulate the expression of catabolic pathways, all of which can be grouped by the more general term of "physiological control".

3.3. Factors Affecting Catabolic Pathways that Rely on σ^{54}-Dependent Promoters

Several catabolic pathways rely on promoters that are recognized by a form of RNA polymerase (RNAP) bound to the alternative sigma factor σ^{54} (or σ^{N}). This form of RNAP is special in many ways. Its ability to initiate

transcription strictly requires the participation of a particular class of transcriptional activators that bind to DNA sites located unusually distant from the promoter core. Most σ^{54}-dependent promoters contain a binding site for the IHF (*i*ntegration *h*ost *f*actor) protein located between the regulator and the RNAP sites. Depending on the promoter considered, IHF helps to stabilize the RNAP at the promoter[4, 47] or enhances transcription initiation at a later step, after RNAP has occupied the promoter[68]. IHF can also improve the specificity of the response by preventing promiscuous cross-activation by other σ^{54}-dependent activators[47]. The most thoroughly studied σ^{54}-dependent catabolic pathways in *Pseudomonas* are the toluene/xylene upper pathway encoded by the *P. putida* TOL plasmid pWW0[51], and the pathway for (methyl)phenol encoded by the *P. putida* CF600 plasmid pVI150[50]. Regulation of these pathways is covered in detail in other chapters of this book, and only a brief discussion of aspects relevant to their modulation by physiological control will be given here. In the TOL pathway, the σ^{54}-dependent promoter *Pu* is controlled by a toluene-responsive transcriptional activator named XylR (see Figure 1B). In the case of the (methyl)phenol pathway, the equivalent transcriptional activator is DmpR, which controls the *Po* promoter in response to phenol and methylphenols (see Figure 1C). XylR and DmpR are structurally and mechanistically closely related proteins. The *Pu* and *Po* promoters also share certain similarity, and both contain an IHF binding site upstream from the core promoter sequences. Induction of the *Pu* and *Po* promoters by the corresponding pathway substrates is tightly modulated by a global control that is dominant over the specific regulators XylR and DmpR, and which responds to the physiological conditions of the cell and/or to the presence of additional preferred carbon sources[20, 23, 24, 31, 41, 69]. This modulation is channelled through several different mechanisms, which do not affect the two pathways to the same extent.

The first type of modulation is evident when cells are grown in a rich medium, a situation in which both the *Pu* and *Po* promoters remain silent during all the exponential phase of growth in spite of the presence of the aromatic inducer in the medium[11, 20, 41, 69]. The repression effect abruptly disappears when cells enter the stationary phase of growth. This behaviour derives from a complex combination of factors. The first one is related to the IHF protein. In both *E. coli* and *P. putida*, the levels of IHF in cells growing in rich medium are much lower during exponential growth than in the stationary phase, so that the IHF site upstream from *Pu* remains empty throughout the exponential phase[74]. Lack of IHF significantly reduces *Pu* activity both in vivo and in vitro, because IHF helps to recruit σ^{54}-RNAP to the promoter[4]. Therefore, it was proposed that IHF couples the transcriptional activity of *Pu* promoter to the growth phase[74]. In the case of the *Po* promoter, IHF does not appear to play a role in recruiting σ^{54}-RNAP, but it has a distinct task in promoting or stabilizing open complexes of σ^{54}-RNAP at the promoter[68].

The *Pu* promoter shows a residual activity in an IHF-deficient strain, and this activity is still growth phase-dependent. Therefore, additional physiological signals are probably entered in the system either through σ^{54}-RNAP or through the activity of the XylR activator. There are at least two ways in which this may occur. The *Pu* and *Po* promoters respond to the nutritional alarmone (p)ppGpp, although *Po* is much more sensitive to it than *Pu*[7, 70]. Synthesis of (p)ppGpp by the ribosome-associated RelA protein is activated by the arrival of uncharged tRNAs to the ribosome and is also produced in response to other stresses by the dual functional SpoT protein[14]. Conditions of nutritional stress that produce a shortage of charged tRNAs signal the cell to down-regulate protein synthesis in accordance to demand. This effect, called the "stringent response," leads to the stimulation of transcription of some promoters and to the inhibition of others. In particular, DmpR activation of the *Po* promoter in the presence of phenols requires (p)ppGpp. The levels of this alarmone are very low when cells grow exponentially in a rich medium, but rapidly increase when cells reach the transition to the stationary phase of growth. Artificial overproduction of (p)ppGpp during exponential growth in a rich medium allowed an immediate response of the *Po* promoter to the presence of phenol[70]. Interestingly, at least in *E. coli* the levels of (p)ppGpp are high during exponential growth in minimal salts medium, which would explain why the *Po* promoter can be efficiently activated by phenols when cells grow exponentially in this kind of medium. (p)ppGpp binds to a site located at the interface of the β and β' subunits of RNAP[15, 72]. In so doing, (p)ppGpp destabilizes the complexes formed at promoters by σ^{70}-RNAP (the major "housekeeping" form of RNAP). Therefore, promoters that form intrinsically unstable complexes with σ^{70}-RNAP, such as the strong stringent *P1* promoters of the *E. coli* rRNA operons, are particularly sensitive to inhibition by (p)ppGpp[2, 3]. Down-regulation of this kind of strong promoters would be predicted to increase the pool of RNAP available for other promoters, as well as the amount of RNAP core enzyme available to be bound by alternative sigma factors. Since sigma factors compete for binding to the RNAP core, which is present in limiting amounts, (p)ppGpp could potentially affect this competition in favour of alternative sigma factors[34, 38]. Several lines of evidence support this idea for the *E. coli* stress-related factors σ^{S} and σ^{H} [34], as well as for σ^{54} [35]. Artificial manipulation of σ-factor levels and/or availability suggests that (p)ppGpp is not an absolute requirement for σ^{54}-dependent transcription of the *Po* promoter. Rather, the elevated synthesis of (p)ppGpp that occurs at the onset of the stationary phase facilitates successful competition of σ^{54} for the available RNAP core, and thus higher levels of this holoenzyme, which in turn results in enhanced activity of the *Po* promoter[35]. Promoters that are easier to saturate with σ^{54}-RNAP would be predicted to be less sensitive to (p)ppGpp. This may be the case of the *Pu* promoter when IHF is at sufficiently high concentrations.

It should be noted that, in vitro, σ^{54}-RNAP has a similar affinity for the *Pu* and *Po* promoters when IHF is present, but when IHF is absent its affinity for *Pu* is significantly lower than for *Po*[68]. Therefore, the main—though not unique—determinant for the repression effect of rich media on the XylR/*Pu* system would be the need for IHF to recruit σ^{54}-RNAP. In the case of the DmpR/*Po* system, IHF is less important but (p)ppGpp is decisive for transcription initiation.

To investigate whether the different sensitivity of the *Pu* and *Po* promoters is due to the promoters, or rather to the corresponding specific activators, regulator-swapping experiments were performed between the XylR/*Pu* and the DmpR/*Po* systems. Perhaps surprisingly, results indicated that the nature of the regulator plays a dominant role in the sensitivity to (p)ppGpp[67]. The authors suggest that the simplest interpretation is that (p)ppGpp binding to the core RNAP, in addition to its effect on σ^{54}-RNAP formation, may also modulate the efficiency with which DmpR interacts with σ^{54}-RNAP to activate transcription.

There is a second way to couple the activity of some σ^{54}-dependent promoters to the physiological status of the cell. *E. coli* contains a protease, named FtsH, which plays a key role in gene regulation in response to diverse physiological signals by controlling the stability of several transcription factors[58, 71]. FtsH is present in many other bacteria, *P. putida* one among them[58, 67]. This protease was found to be essential for the expression of *Pu* and three other σ^{54}-dependent promoters in *E. coli*[6]. Cells lacking FtsH express σ^{54} at normal levels, but *Pu* and some other σ^{54}-dependent promoters behave as if this sigma factor was inactive. Overproduction of σ^{54} restored *Pu* activity to a large extent in a FtsH-deficient *E. coli* strain, which was taken as an indication that FtsH could control the activity of σ^{54}. However, the finding that activation of the σ^{54}-dependent *Po* promoter from the (methyl)phenol degradation pathway is efficient in the absence of FtsH led to analyze this problem with a different approach[67]. Regulator swapping experiments between the XylR/*Pu* and DmpR/*Po* systems indicated that the regulator, rather than the promoter or σ^{54}, determines sensitivity to the absence of FtsH[67]. Besides catalyzing degradation of proteins, FtsH may also act as a molecular chaperone[58], assisting in the assembly, operation or disassembly of protein complexes. In response to a proper signal, for example, detection of toluene or phenols in the case of XylR or DmpR, σ^{54}-dependent regulators oligomerize and form higher order structures to activate transcription. It is possible that FtsH may be required for correct folding or for proper assembly and/or disassembly of regulator multimers or regulator–σ^{54}-RNAP complexes. Unlike XylR and other σ^{54}-regulators, DmpR can readily oligomerize in the absence of DNA binding[77]. Although unknown, this may underlie why XylR requires FtsH while DmpR does not[67].

The activity of promoter *Pu* is also down-modulated by a different mechanism that responds to the presence of glucose or other carbohydrates.

The activities of the PtsN (also named IIA^{Ntr}) and PtsO (or NPr) proteins are important for this control[12, 13]. These proteins show similarity to phosphotransferases of the PTS family, particularly to members of the IIA and HPr subfamilies, respectively. Although the PTS system is associated in many bacteria with the transport of sugars across cell membrane coupled to their phosphorylation, the *P. putida* PtsN and PtsO proteins do not seem to participate in glucose transport. The corresponding genes, *ptsN* and *ptsO*, belong to a gene cluster that also includes *rpoN*, the gene that encodes σ^{54}. These three genes are coexpressed. Inactivation of *ptsN* makes *Pu* unresponsive to repression by glucose, although glucose consumption remains unaffected[12]. On the contrary, inactivation of *ptsO* results in inhibition of *Pu* activity even in the absence of glucose[13]. The activity of PtsN and PtsO seems to rely on their phosphorylation status. All these data suggest that PtsN and PtsO belong to a signal transmission pathway that inhibits *Pu* induction by toluene or xylene when glucose or other preferred carbohydrates are present. However, the precise mechanism responsible for this repression is still unknown. It should be noted that the *Po* promoter of the (methyl)phenol pathway is not influenced by PtsN-mediated glucose repression[67, 69]. Again, regulator-swapping experiments between the XylR/*Pu* and the DmpR/*Po* systems showed that the susceptibility to glucose repression relies on the identity of the regulator, so that PtsN probably functions by affecting the activity of XylR[67]. It is worth noting that the glucose-mediated repression of promoter *Pu* is genetically distinguishable from the repression effect observed in rich medium, since inactivation of the *ptsN* gene does not alleviate the repression in rich medium[9].

It is still unclear whether the XylR/*Pu* and the DmpR/*Po* systems are sensitive to Crc and to the cytochrome *o* ubiquinol oxidase. However, succinate repression of the phenol pathway encoded in the *P. putida* H pPGH1 plasmid is at least partially mediated by this oxidase[48]. Expression of this phenol pathway also depends on σ^{54}, and the specific transcriptional regulator (PhlR) is closely related to DmpR and XylR. On the other hand, the repression generated by excess succinate on expression of the XylR/*Pu* system decreases considerably when O_2 is limited[24, 25]. Under these conditions, expression of the cytochrome *o* ubiquinol oxidase decreases considerably[21]. This suggests the cytochrome *o* ubiquinol oxidase could also have some role in the repression of other σ^{54}-dependent pathways, although the diversity of mechanisms observed to date impedes the drawing of general conclusions.

3.4. Direct Mechanisms

At least in one case no intermediate factors are needed to transform the presence of the repressing carbon source into a regulatory signal. The *clc* operon present in the *P. putida* pAC27 plasmid encodes the genes necessary

for the assimilation of 3-chlorocatechol via 2-chloro-*cis,cis*-muconate. Expression of the *clc* genes requires the transcriptional activator ClcR and 2-chloro-*cis,cis*-muconate, which acts as effector. Induction of the pathway is repressed when cells are grown in the presence of succinate, citrate or fumarate, all of which are intermediates of the tricarboxylic acid cycle. The effect was traced to the ClcR activator, which was unable to activate transcription in the presence of high fumarate concentrations[42]. In vitro assays showed that the effect of fumarate is concentration dependent and reversible. It was therefore proposed that fumarate and 2-chloro-*cis,cis*-muconate compete for binding to ClcR, so that when the effector pocket is occupied by fumarate ClcR is unable to activate transcription. Since fumarate is a component of the tricarboxylic acid cycle, its binding to ClcR provides a direct connection between the metabolic status of the cell and the repression of a catabolic pathway for a non-preferred substrate.

CONCLUSIONS

The examples presented show that the induction of catabolic pathways in Pseudomonads is carefully modulated by diverse factors, a property that probably helps them to compete and survive in complex and changing environments. The nutritional state of bacteria can change from a situation of nutritional excess, to another one of total starvation, with an intermediate situation where the concentration of certain nutrients is suboptimal. This intermediate situation is probably the most frequent circumstance encountered in nature. It has been termed as the "hunger" condition, and is in many respects different from starvation[26]. Each situation has a specific global regulation response that can significantly affect the induction of many genes, and of catabolic pathways in particular. The examples described above show that induction of catabolic pathways in *Pseudomonas* can be influenced not only by the presence of alternative carbon sources, but by other environmental or physiological signals such as oxygen availability or responses elicited by the transition from a feast to hunger situation (see Figure 3). Several factors have been identified that can sense and transmit this information, although the final picture is far from being complete. Crc seems to sense signals from carbon metabolism only, while the cytochrome *o* ubiquinol oxidase, by gathering information on the activity of the electron transport chain, can check both oxygen availability and what type of carbon source is being used. Other factors such as IHF or the nutritional alarmone (p)ppGpp, that can have a great impact on the modulation of some pathways, are more characteristic of the hunger response. Clearly, different combinations of factors can influence each catabolic pathway. The net

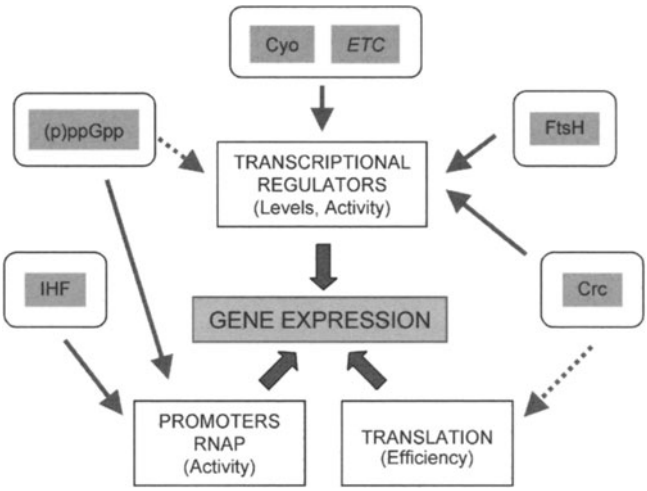

Figure 3. Summary of factors modulating the activity of catabolic promoters. Only those factors commented in the text are indicated. Discontinuous lines indicate effects that remain to be rigorously proven. RNAP, RNA polymerase; ETC, electron transport chain.

result is that a particular catabolic promoter can be regulated by a network of global regulatory signals, although not all of them have the same influence.

A particularly interesting example is the repression observed in cells growing exponentially in rich medium, which affects pathways that are regulated through very different mechanisms. This response arises from the integration of several signals. It is observed only during exponential growth on rich media, and is abruptly relieved when cells adapt to enter the stationary phase. At this critical point cells probably sense different signals such as consumption of the preferred carbon sources being used (although other potential carbon sources remain in the medium), a decrease in oxygen availability, an increase in cell density, modifications in pH, etc. Some of these signals are hunger signals, but others are not. All this information reaches different pathways through different global regulators. In the case of the *P. putida* alkane degradation pathway, the repression induced by rich medium is mediated to a large extent by Crc and by the cytochrome *o* ubiquinol oxidase. However, at the *P. putida* XylR/*Pu* system from the toluene/xylene pathway this type of repression depends mainly on IHF and, to a lower extent, on (p)ppGpp. Finally, at the *P. putida* DmpR/*Po* system from the (methyl)phenol pathway the same information is channelled primarily through (p)ppGpp, with IHF playing a lesser role. Clearly, there are several ways to reach the same final regulatory response. It could be expected that all these strategies are effective enough

so far as they ensure both a suitable responsiveness to the pathway substrate and a proper connection to the physiological state of the bacteria[10]. It can be argued whether the repression effect observed in rich media is a classical catabolite repression phenomenon, or should be better described under the more general term "physiological control." Since this response results from the integration of several different signals, not all directly connected to carbon metabolism, it is reasonable to suggest that it is not just carbon catabolite repression, but something more complex that is best described as "physiological control."

Although the number of examples analyzed in detail is small, Pseudomonads global regulatory systems interfere with the induction of catabolic pathways by predominantly exploiting properties of the specific transcriptional regulators of each pathway. In some cases the regulation is targeted to down-modulate the levels of the specific transcriptional regulator of the pathway. This is the case of AlkS from the OCT plasmid alkane degradation pathway, or of BkdR from the *bkd* operon. In other cases, it is the ability of the regulator to regulate transcription, rather than its levels, that is the target of the physiological control. Clear examples are the XylR activator from the pWW0 toluene pathway, the DmpR activator from the pVI150 phenol pathway and the ClcR activator from the pAC27 chlorocatechol pathway. Global regulation systems have other targets as well, as exemplified by the need of IHF for efficient recruitment of RNAP at the *Pu* promoter from the pWW0 toluene upper pathway, or the proposed interference of the Crc protein in the translation process. Exploitation of the distinct properties of the specific regulators and promoters of each pathway, as well as other aspects of gene expression, could provide substantial diversity to physiological control mechanisms. However, more knowledge on the mechanisms mediating global control of the catabolic pathways of *Pseudomonas* sp. is required before final conclusions can be drawn. Identification of all the components mediating the regulation effects, and the possible connections and/or hierarchies among them, is an important aspect to elucidate in the next future. The possible role of small non-coding RNAs (sRNAs) on the physiological control of carbon metabolism in Pseudomonads is another promising line of research. Close to 50 sRNAs have been found in *E. coli*, where they play diverse roles coordinating and regulating the expression of many genes in response to environmental changes[52, 75]. Some of them are directly involved in global regulation of carbon metabolism (ref. [76] and references therein). Information on the role of sRNAs in Pseudomonads is very scarce, but it will not be surprising to find that they also play an important role in many aspects of carbon metabolism and on the physiological control of catabolic pathways. There is clearly much to be done yet to fully understand the global regulation of carbon metabolism in Pseudomonads.

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14

REGULATION OF *PSEUDOMONAS AERUGINOSA* EXOTOXIN A SYNTHESIS

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1. THE ROLE OF EXOTOXIN A IN THE PATHOGENESIS OF *PSEUDOMONAS AERUGINOSA* INFECTION

Exotoxin A (ETA) is one of the most toxic virulence factors produced by *Pseudomonas aeruginosa*[72]. Both clinical and experimental animal studies indicate clearly the importance of ETA in the pathogenesis of different *P. aeruginosa* infections. Most *P. aeruginosa* clinical isolates produce ETA[9, 23, 47]. In addition, infection with ETA-producing *P. aeruginosa* strains was shown to be significantly associated with mortality due to *P. aeruginosa* bacteremia[98]. Furthermore, survival of patients with *P. aeruginosa* bacteremia correlated with high ETA antibody titers in those patients[98, 99]. Examination of patients with cystic fibrosis (CF) that were chronically infected with *P. aeruginosa* showed the presence of an increased titer of ETA antibody[57]. In addition, mortality among *P. aeruginosa* infected CF-patients was associated with increased levels of ETA and LPS antibodies together with the presence of immune-complexes and complement activation[84]. Recent analysis of RNA extracted

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from sputa of CF patients revealed that the ETA gene (*toxA*) was expressed in the lung of CF patients infected with *P. aeruginosa*[105].

The role of ETA in *P. aeruginosa* infections has also been demonstrated by other in vivo and in vitro studies. Intravenous injection of mice with purified ETA resulted in a high mortality rate, apoptosis of the hepatocytes, and the enhanced expression of several proinflammatory cytokines[36, 110]. These effects were eliminated when the mice were pretreated with specific ETA-antibody[36]. ETA also enhances the growth of virulent *P. aeruginosa* in vivo, possibly by decreasing the number of the polymorphonuclear leukocytes (PMNLs)[56, 87]. Among the in vitro effects of ETA are lethality to both human and murine PMNLs and inhibition of phagocytosis and killing of *P. aeruginosa* by the PMNLs[82]. ETA also decreases the production of tumor necrosis factor, lymphotoxin, and interferon-γ in lymphocytes or macrophages and the production of interleukin 1β (IL-1β) and IL-1α[113]. In the thermally injured mouse model, the survival of mice infected with *P. aeruginosa* was enhanced upon the intravenous injection of ETA-antibody[94]. In comparison with their parent strains, the lethality among thermally injured mice infected with ETA-deficient mutants of *P. aeruginosa* was significantly lower[36, 86]. In the murine model of keratitis, purified ETA induced structural ocular damage that was similar to the damage produced during experimental *P. aeruginosa* keratitis[52]. In addition, *P. aeruginosa* ETA-deficient mutants produced less ocular damage than their parent strains[92, 96]. The mutants were similar to their parent strains in their adherence to the wounded corneal tissue[96]. However, in contrast to their parent strains, the mutants were quickly cleared from the eyes and caused no corneal damage[96].

2. THE STRUCTURE AND FUNCTIONS OF EXOTOXIN A AND ITS DOMAINS

The toxicity of ETA is due to its ability to irreversibly inhibit protein synthesis within its target eukaryotic cells. The predominant enzymatic activity of ETA is its ADP-ribosyl transferase activity[60, 61]. In this activity, ETA catalytically transfers the ADP-ribose moiety of NAD^+ onto elongation factor 2 (EF2)[127, 138]. This leads to the cessation of polypeptide chain elongation and inhibits protein synthesis[127, 138]. When its EF2 substrate is not available, ETA exhibits NAD-glycohydrolysis activity that hydrolyzes NAD^+ into nicotinamide and ADP-ribose[18, 69].

ETA is synthesized as a 71-kDa precursor with a typical 25-amino acid leader peptide at its amino terminus end[43]. During ETA secretion through the type II secretion pathway[30, 131], the leader peptide is removed and the mature 66.5 kDa protein is released into the extracellular environment[30, 34, 43, 103]. The mature ETA is toxic to both animals and cell lines but is enzymatically

inactive[18, 69, 127]. In vitro activation of ETA is usually achieved by treating the molecule with both denaturing and reducing agents such as urea and DTT, respectively[18, 127, 140]. This treatment modifies the ETA molecule by denaturing it and reducing some or all of the four disulfide bonds located within the amino terminus region of ETA[127, 140]. ETA can also be activated by proteolytic treatment that releases the enzymatically active part (fragment) of the molecule[75, 127, 140].

Initial steps in ETA interaction with the eukaryotic cell involve binding to a specific cell surface receptor[37, 123]. Earlier studies indicated that ETA binds to a high molecular weight cell surface glycoprotein[37, 123]. Kounnas *et al.* later identified the ETA receptor as the α2-macroglobulin receptor/low density lipoprotein receptor-related protein, which is found in many tissues[65]. This receptor is highly expressed in both fibroblasts, which are sensitive to ETA, and hepatocytes; most of the in vivo toxicity of ETA is accomplished through liver damage[65, 79, 110, 112]. After binding, ETA is internalized via clathrin-coated pits into endocytic vesicles (endosomes)[35, 83, 108]. Within the vesicles, ETA is cleaved by a cell-associated protease into a 28-kDa amino terminus fragment and a 37-kDa carboxy terminus fragment[91]. The 37-kDa fragment, which contains the ADP-ribosylating activity, is then translocated into the cytosol[91]. The translocation process requires a low pH[1, 32, 62]. This low pH is produced by the acidification of the endosomes through the proton pumps within the membrane[1, 32, 62]. The low pH is required for the translocation of the cleaved 37-kDa fragment but not for the proteolytic process itself[91].

Based on the crystallographic analysis of the proenzyme form of ETA and analyses of the deduced amino acid sequence of the protein, Allured *et al.* proposed that ETA is composed of three functional domains (I, II, and III)[3]. Domain I is composed of two subdomains; Ia (residues 1–252) and Ib (residues 365–404)[3, 43]. The subdomains are adjacent to each other within the crystalline structure[3, 43]. Domain II consists of residues 253–364 and domain III consists of residues 405–613[3, 43]. Hwang *et al.* utilized this information to determine the function of each domain by separately cloning the regions of *toxA* that code for each domain in an expression vector and examining the biological activity of the peptides encoded by each clone[59]. Both in vitro and in vivo analyses confirmed that domain Ia is the receptor-binding domain[59]. Additional studies, including deletion analysis, chemical modification of specific residues, and oligonucleotide-directed mutagenesis, confirmed that domain Ia binds to the receptor and identified several residues within the domain that are critical for this binding[16, 44, 63, 97]. The function of domain Ib is not clear. Siegall *et al.*[111] showed that an ETA recombinant protein in which the amino terminus portion of domain Ib (aa 365 – 380) was deleted retained full ADP-ribosylating activity and was slightly more cytotoxic. Hwang *et al.* also showed that domain II is required for ETA translocation into the cytosol[59].

Additional studies identified specific regions within domain II that play an important role in ETA translocation[1, 122]. Domain III catalyzes the ADP-ribosylation of EF2[59]. The role of different residues within domain III in the ADP-ribosylating activity was extensively analyzed. Results of those studies suggested that glutamine at position 553 (gln-553) functions as a nicotinamide subsite of NAD^+ binding while arg-458 and arg-469 play a role in the interaction of domain III with NAD^+ (see refs [11], [78], [140]). In addition, tyr-481 and his-426 may be involved in aspects of the ADP-ribosylation reaction other than NAD^+ binding[77, 139, 140].

3. REGULATION OF EXOTOXIN A SYNTHESIS IN *P. AERUGINOSA*

3.1. Environmental Factors Affecting Exotoxin A Production

Exotoxin A production in *P. aeruginosa* is controlled by different environmental stimuli. Optimum levels of ETA are produced in vitro when *P. aeruginosa* is grown to late growth phase at 32°C with maximum aeration[73]. The best medium for ETA production is dialyzed trypticase soy broth (TSB-DC) supplemented with 1% glycerol as a carbon source and monosodium glutamate[73]. The addition of heat-treated nucleic acids or nondialyzed TSB enhances the growth of *P. aeruginosa* but inhibits ETA production[73]. ETA production is also inhibited by the presence of different cations in the growth medium[8, 26]. The single cation with the most profound effect on ETA production is Fe^{2+} (see ref. [8]). The presence of $FeCl_3$ in the growth medium at a concentration of 5 μM or higher severely reduces ETA production[8]. Therefore, dialyzed TSB is usually Chelex®-treated to remove most of its iron content. Blumentals *et al.*[10] studied the effect of different cations on ETA production by utilizing a modified defined medium and a two-step process to determine the effect of different cations on ETA production by *P. aeruginosa*. The two-step process was included to separate bacterial growth from ETA production[10]. In the first step, *P. aeruginosa* was grown to stationary phase in modified minimal medium and the cells were washed to remove any residual iron; then in the second step, cells were resuspended in the modified minimal medium containing no iron and grown for 4–6 hr[10]. Their results showed that besides Fe^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+} inhibit ETA production[10]. In contrast, Ca^{2+} at concentrations up to 500 μM enhanced ETA production[10]. However, the presence of Ca^{2+} did not interfere with the inhibitory effect of Fe^{2+} on ETA production[10].

3.2. The *toxA* Gene

The structural gene for ETA (*toxA*) was originally cloned in *Escherichia coli* from a gene bank of the *P. aeruginosa* strain PA103[17, 43]. Nucleotide sequence analysis of the cloned gene showed that *toxA* codes for a 71-kDa precursor protein[43]. Transcriptional studies using S1 nuclease experiments revealed the presence of two *toxA* transcripts that initiate at 89 and 62 bp 5′ of the ETA initiation codon, respectively[17, 41]. The sequence within the −10 and −35 portion of each transcript showed no homology to the consensus −10 and −35 regions of prokaryotic promoters[41]. The 5′ end of the smaller transcript is located 8 bp upstream of a dodecamer sequence that is conserved within the upstream region of *toxA* and the *P. aeruginosa* phospholipase C gene[41]. To examine the role of different fragments of the *toxA* upstream region in *toxA* expression, Tsaur and Clowes[124] constructed several *Bal*31 deletions within the upstream region of the PA103 *toxA* gene and replaced these deletions with an *Eco*RI linker sequence. Analysis of these constructs revealed that the region 75 bp upstream of the *toxA* transcription initiation site (160 bp at 5′ of the initiation codon) is sufficient for *toxA* expression in *P. aeruginosa*[124]. In addition, nucleotide substitution at two specific sequences located at either −2 to −9 bp or −54 to −72 bp at 5′ with respect to the *toxA* transcription initiation site reduced *toxA* expression to less than 5%[124]. Tsaur and Clowes suggested that the −2 to −9 bp region contains a putative RNA polymerase binding site while the −54 to −72 bp region represents a binding site for a potential *toxA* transcriptional activator[124]. In the absence of such an activator, as in *E. coli, toxA* transcription does not occur. Further studies showed that certain *P. aeruginosa* genes (including *toxA*) are not transcribed in *E. coli* because the *E. coli* RNA polymerase does not recognize their promoters[41, 76, 126]. *toxA* transcription in *E. coli* is detected only when *toxA* is expressed from a promoter recognized by the *E. coli* RNA polymerase[41, 49, 76].

Shortly after *toxA* isolation, RNA analysis revealed that ETA is regulated by iron at the transcriptional level[74]. In an iron-deficient medium, ETA synthesis, as well as the accumulation of *toxA* mRNA, increased at late log and early stationary phase of growth[74]. However, in the presence of iron, no *toxA* mRNA was detected[74]. When *P. aeruginosa* was grown in media that allow ETA synthesis, the half-life of *toxA* mRNA varied between 6 and 10 min[74]. Upon the addition of iron to a *P. aeruginosa* culture that was actively synthesizing ETA, *toxA* mRNA decreased slowly (a half-life of 90 min) with a long lag period between the addition of iron and the detection of the effect[74]. During this delay, cellular components that are necessary to relay the change in the level of iron to the transcriptional apparatus were thought to be synthesized[74].

3.3. Genes Involved in *toxA* Regulation

The regulation of ETA production by different environmental conditions suggested that *P. aeruginosa* utilizes several regulatory genes to fully control *toxA* expression. Throughout the years, several *P. aeruginosa* genes that regulate *toxA* expression either positively or negatively have been identified. Among the positive *toxA* regulatory genes are *regAB, pvdS, ptxR*, and *vfr* (virulence factors regulator); among the negative regulatory genes are *fur* and *ptxS*. The efficiency with which *toxA* positive regulators affect *toxA* expression varies. For example, *regAB* is essential for *toxA* expression while *ptxR* and *vfr* are not. No *toxA* expression was detected in a *P. aeruginosa* strain that carries a deletion within *regAB*. However, *toxA* expression is only reduced in *P. aeruginosa* strains that carry a deletion within either *ptxR* or *vfr*. A similar phenomenon was detected with respect to the negative regulation of *toxA*; *fur* represses *toxA* expression very efficiently while *ptxS* represses *toxA* expression by only 2 fold. In comparison with other *toxA* regulatory genes, *regAB* appears to be designed mainly to regulate *toxA* expression while *pvdS, fur*, and *ptxR* regulate the expression of both *toxA* and the siderophore genes. In addition, *vfr* regulates the expression of the quorum sensing gene *lasR* and *ptxS* regulates the expression of the *kgu* operon. Similar to the regulation of other prokaryotic virulence genes, the regulation of *toxA* expression in *P. aeruginosa* is accomplished through a hierarchical system that involves several intermediate regulators. Despite extensive analysis, the exact mechanisms through which *toxA* expression is regulated by these different genes are not completely understood. Below, we provide the current understanding of the relationship between *toxA* and each of the above-mentioned regulatory genes, which are summarized in Table 1.

Table 1. A summary of the types of *toxA* regulatory proteins and their target genes.

Regulatory protein	Type/family	Target genes
Positive regulators		
RegA	No special family	*toxA, tol–oprL*
RegB	No special family	*regAB* (?)
PvdS	Alternative sigma factor; Extracytoplasmic factors	*pyoverdine genes, toxA, regA, ptxR*
PtxR	LysR transcriptional activators	*toxA, regA, pvc*[a]
Vfr	Catabolite repressor proteins	*lasR, ptxR, toxA, regA*
Negative regulators		
Fur	Ferric uptake regulators	*pvdS, toxA, regA*, ~30 other genes (PIGS)[b]
PtxS	GalR transcriptional repressors	*kgu*[c], *ptxR*

[a]*pvc*, pyoverdine chromophore synthesis operon.
[b]PIGS, *P. aeruginosa* iron-regulated genes.
[c]*kgu*, 2-ketogluconate utilization operon.

3.3.1. The regAB *Locus*

3.3.1.1. *regA.* Ohman *et al.* have previously described the isolation of several chemically induced ETA-deficient mutants of PA103[93]. Using PA103-29, one of the ETA-deficient mutants, as a host for complementation analysis, Hedstrom *et al.*[53] screened a PA103 gene bank to isolate a potential *toxA* regulatory gene. The presence of the isolated clone in different *P. aeruginosa* strains (including PAO1, PA103, and PAK) enhanced ETA synthesis by about 10 fold and partially alleviated the inhibitory effect of iron on ETA production[53]. Further subcloning and complementation analyses localized the effect on ETA synthesis to a 1.9-kb fragment[55, 144]. Nucleotide sequence analysis revealed the presence of an ORF encoding a 29-kDa protein, RegA (also called ToxR)[54, 144]. Immunoblotting experiments using RegA-specific polyclonal antibody confirmed that RegA is membrane associated in strain PA103[145]. The predicted amino acid sequence of RegA contains a transmembrane α-helix at the amino terminus region[104]. Initial studies clearly showed that RegA enhances ETA production at the transcriptional level (see below). Neither ETA nor *toxA* mRNA was detected in the *regA*-deficient mutant PA103-29[39]. However, despite several subsequent studies, the exact mechanism through which RegA enhances *toxA* transcription remains unknown. Computer analysis revealed no significant homology between the predicted amino acid sequence of RegA and other prokaryotic transcriptional activators[48]. In addition, RegA synthesized in *E. coli* from the T7 promoter did not bind to the *toxA* upstream region[48]. Walker *et al.* utilized purified T7-produced RegA (ToxR) plus purified *P. aeruginosa* RNA polymerase (Pa-RNAP) in run-off transcription assays to examine RegA function[132]. Pa-RNAP alone failed to initiate *toxA* transcription[132]. However, *toxA* transcription was detected when Pa-RNAP and purified RegA were added to the reaction[132]. In vitro *toxA* transcription was enhanced upon the addition of a *P. aeruginosa* lysate fraction (either PA103 or PA103-29) to the Pa-RNAP and purified RegA[132]. The ability of PA103-29 lysate to enhance *toxA* transcription suggests the presence of a *regA*-independent *toxA* regulator[132]. In addition, specific binding between the purified RegA and the Pa-RNAP was demonstrated by several experiments. Based on their evidence, Walker *et al.* suggested that RegA is an alternative sigma factor that associates with the *P. aeruginosa* RNAP and initiates *toxA* transcription[132]. If RegA is an alternative sigma factor, the relevance of its association with the *P. aeruginosa* membrane is not clear. Additional run-off transcription assays conducted in the presence of heparin demonstrated that the *P. aeruginosa* RNAP requires purified RegA to form an open complex (heparin sensitive) with the *toxA* upstream region[131]. Even the heterologous *E. coli* RNA polymerase transcribed *toxA* in the presence of purified RegA[131]. Walker *et al.* utilized Pa-RNAP, purified RegA, and DNA fragments carrying different parts of the *toxA* upstream region in additional in vitro transcription

assays to identify the specific sequence within the *toxA* upstream region that plays a role in the regulation of *toxA* transcription by RegA[131]. The smallest fragment that produced a *toxA* transcript in the presence of RegA was a 214 bp *Pvu*II/*Bam*HI fragment[131]. This fragment contains the 20 bp *toxA* activator-binding site previously proposed by Tsaur and Clowes[124, 131].

Due to the lack of homology between RegA and other prokaryotic transcriptional activators, a potential RegA functional domain could not be identified[48]. Raivio *et al.* later utilized 6-bp linker insertion mutagenesis in their functional analysis of RegA to address this problem[104]. Several RegA-linker insertion mutants in which the effect on ETA production was compromised to varying degrees were characterized[104]. A linker insertion at amino acid (aa) 88 greatly reduced its effect on ETA production[104]. In addition, the production of this reduced level of ETA was deregulated with respect to iron[104]. This insertion was located upstream of the predicted transmembrane α-helix[104]. Additional linker insertions between aa 88 and aa 163 reduced the effect of RegA on ETA production by 6–7 fold throughout the growth cycle of PA103[104]. Computer analysis revealed that the region defined by these linker insertions (aa 87 to aa 142) has 42% homology to the transcriptional activation domains of *Vibrio cholerae* ToxR, *Agrobacterium tumefaciens* VirG, and *E. coli* OmpR[80, 100, 104, 109]. The transcriptional activation domains are involved in the binding of these three regulators to their target sequences as well as in the binding of two of the regulators (VirG and OmpR) to the RNA polymerase[31, 125]. Results of the linker insertion mutagenesis of RegA supported the previous suggestions by Walker *et al.*[131, 132] that RegA interacts with the Pa-RNAP. Based on the conclusions of these two studies, it would be interesting to examine the effect of purified RegA proteins carrying the 6-bp linker insertions on in vitro transcription of *toxA* by the Pa-RNAP and the possible interaction of these RegA proteins with the Pa-RNAP.

3.3.1.2. *regB.* The level of ETA produced by the PA103 strain is usually about 10 fold higher than that produced by strain PAO1[138]. This variation may be caused by differences between the two strains in the *toxA* regulator, RegA. To examine such a possibility, Wick *et al.* isolated the DNA fragment that carries the PAO1 *regA* gene[137]. Comparison of the nucleotide sequences of the DNA fragment that carries PA103 *regA* with the one that carries PAO1 *regA* revealed several differences[137]. Three single base pair differences were detected within the *regA* open reading frame (ORF); however, only one resulted in an amino acid substitution—alanine at position 144 in PA103-RegA to threonine in PAO1-RegA[137]. The most significant difference noted was a mutation in the PAO1-*regA* locus for the initiation codon of a small ORF that follows *regA* in the PA103 locus[137]. This ORF (*regB*) is located 6 bp at 3′ of the *regA* stop codon and codes for a 7.5 kDa predicted protein[117].

In PA103, *regB* is transcribed together with *regA*. Thus, *regA* and *regB* are considered to constitute an operon, the *regAB* locus[137, 138]. In PAO1, the *regAB* locus encodes RegA only due to the mutation in the *regB* initiation codon. To examine the importance of *regB*, Wick *et al.* constructed a hybrid *regAB* locus in which the PA103-*regB* was substituted with the PAO1-*regB*[137]. The level of ETA produced by the *regA* mutant (PA103-29) carrying this hybrid locus was significantly lower than that produced by PA103-29 carrying the intact PA103-*regAB* locus[137]. Although *regB* produces a 7.5-kDa translational product[25], the exact mechanism through which *regB* affects ETA synthesis in *P. aeruginosa* is not known. Available evidence suggests that RegB modulates the expression from one of the *regAB* promoters (see below).

3.3.1.3. Regulation of the *regAB* Locus. Similar to *toxA, regAB* expression is repressed by iron[38]. Earlier studies showed that under iron-deficient conditions, *toxA* and *regA* expression throughout the growth cycle of the *P. aeruginosa* strain PA103 follows a biphasic curve consisting of early and late phases[38, 40]. The early phase is not tightly regulated by iron while the late phase is stringently regulated by iron. The early phase is characterized by the production of a large transcript (T1) that includes mRNA for both *regA* and *regB*; whereas the late phase is characterized by a smaller transcript (T2) that includes mRNA for *regA* only[38, 40]. Further analysis revealed that the *regAB* locus is expressed from two separate promoters; P1, located 164 bp upstream of the initiation codon, codes for the early iron-independent transcript T1 while P2, located 45 bp upstream of the initiation codon, codes for the late iron-regulated transcript T2[116]. Using *regA* transcriptional/ translational fusions, Storey *et al.*[117] analyzed in detail the expression of the P1 and P2 promoters throughout the growth cycle of three specific *P. aeruginosa* strains that carry intact *regAB* (PA103), nonfunctional *regAB* (PA103-29), or nonfunctional *regB* (PAO1)[43, 117, 137]. To determine the effect on the expression from both promoters, a plasmid carrying a P1 + P2 translational fusion was introduced into the three strains. As expected, in PA103, *regAB* expression from this plasmid occurred early in the growth cycle and in both iron-deficient and iron-sufficient media[117]. In PAO1 and PA103-29, however, *regAB* expression from this plasmid was not detected until late in the growth cycle and in iron-deficient medium only[117]. This suggested that in PAO1 and PA103-29 the *regAB* P1 promoter is not expressed[117]. In addition, in PA103, *regAB* expression from the *regAB* P1 promoter was detected immediately after subculturing and continued to increase for 6 hr in iron-deficient or iron-sufficient media whereas in PAO1, no *regAB* expression was detected in either medium[117]. To further support these findings, complementation analysis of PA103-29 with a plasmid carrying either the PA103 *regAB* locus or a hybrid PA103-*regA*/PAO1-*regB* locus was conducted[117]. Whereas the

T2 transcript encoded by the P2 promoter was detected in PA103-29 carrying either plasmids, the T1 transcript encoded by the P1 promoter was detected in PA103-29 carrying PA103 *regAB* locus but not the hybrid locus[117]. These results clearly indicate that the function of RegB is to enhance *regA* transcription from the P1 promoter. Based on this scenario, the *regAB* locus would autoregulate its expression through RegB[117].

3.3.1.4. Other Genes Regulated by *regAB*. The *regAB* locus does not appear to be a global regulator in *P. aeruginosa*. Rather, its effect seems to be localized to *toxA*. With the exception of two studies, no effect of the *regAB* locus on the expression of *P. aeruginosa* genes other than *toxA* has been documented. Wolz *et al.*[143] utilized two-dimensional protein gel analysis and a PA103 mutant that carries an internal deletion within *regA* to determine if RegA regulates the production of other *P. aeruginosa* proteins. No difference was detected in the protein profile of the whole cell lysates of the PA103 *regA* mutant and that of the mutant complemented with a *regA* plasmid[143]. However, comparison of the protein profile of the supernatant fractions of the two strains detected a 41-kDa protein produced by the complemented strain only[143]. The production of this protein was negatively regulated by iron[143]. Using the amino acid sequence, we have searched the *P. aeruginosa* PAO1 genome project (http://www.ncbi.nlm.nih.gov) to try to identify this protein. The gene product is listed as hypothetical protein PA2377; a protein with no significant homology to other previously identified prokaryotic proteins. With the availability of the PAO1 genomic sequence, it would be interesting to compare the global gene expression between PAO1 and its isogenic mutant PAO1::*regA* using microarray analysis. The second, more recent study by Duan *et al.*[29] suggested that RegA positively regulates the expression of the *P. aeruginosa tol–oprL* region, which is required for transporting pyocyanin and maintaining the integrity of the outer membrane[27, 70]. However, unlike its regulation of *toxA* expression, RegA is not essential for the expression of the *tol–oprL* region[29]. Under iron-deficient conditions and at the late stationary phase of growth, the expression of several *tol–oprL* genes was detected in both PA103 and the *regA* mutant, although the level of this expression in the *regA* mutant is considerably lower than that in its parent strain[29].

3.3.2. pvdS

PvdS was originally described as a novel alternative sigma factor belonging to the family of extracytoplasmic factors that positively regulates expression of the genes that code for the *P. aeruginosa* siderophore pyoverdine (*pvd*)[24, 67, 68, 81, 130]. PvdS binds to both a specific target sequence (the iron starvation [IS] box) within the upstream regions of the *pvd* genes and to the *P. aeruginosa* RNA polymerase to initiate transcription of these genes[142].

Besides the *pvd* genes, PvdS positively regulates the expression of *toxA* and *prpL*, the gene that encodes the endoprotease PrpL[87, 141]. Ochsner *et al.*[87] examined the effect of PvdS on ETA production in PAO1 and the mechanism through which this effect is accomplished. Other studies had shown that the *P. aeruginosa* ferric uptake regulator (Fur) regulates *toxA* and *regAB* expression under microaerobic conditions only (see below). Therefore, Ochsner *et al.* compared the level of ETA and *toxA* and *regAB* mRNA between PAO1 and its *pvdS* isogenic mutant grown under aerobic and microaerobic conditions[87]. At stationary phase under both aerobic and microaerobic conditions, the PAOl::*pvdS* mutant produced significantly lower levels of ETA (between 16 and 32 fold less) than its parent strain[87]. However, the reduced level of ETA production by PAO1::*pvdS* was still negatively regulated by iron[87]. RNase protection experiments (primer extension analyses) showed that PAO1 grown aerobically in iron-deficient medium produced *toxA* transcript while PAO1::*pvdS* did not[87]. Similarly, in both aerobic and microaerobic conditions and in iron-deficient medium, the *regAB* T1 and T2 transcripts were detected in PAO1 but not PAO1::*pvdS*[87]. These results demonstrated that in *P. aeruginosa* *toxA* expression is regulated by PvdS through *regAB*. To determine if iron regulates *toxA* and *regAB* transcription through *pvdS*, the effect of a plasmid in which *pvdS* was overexpressed from the *tac* promoter on *toxA* and *regAB* transcription in PAO1 and PAO1::*pvdS* was examined[87]. In this plasmid, *pvdS* expression was iron-independent but inducible by IPTG[87]. Under aerobic and microaerobic conditions, *pvdS* overexpression in PAO1 deregulated ETA production with respect to iron[87]. A similar deregulation occurred in PAO1::*pvdS* that was grown microaerobically but was seen to a lesser degree aerobically; that is, iron repressed ETA production by only 2 fold[87]. The reason for this regulation in PAO1::*pvdS* is unclear but it suggests the possible existence of iron- and oxygen-dependent negative regulation that affects *toxA* expression independently of *pvdS*. The effect of *pvdS* overexpression on *toxA* and *regAB* expression in PAO1 in the presence of iron was also examined[87]. Under aerobic conditions, *toxA* transcription was detected at the early (4 hr) but not the late (8 hr) exponential phase of growth; whereas, under microaerobic conditions, *toxA* transcription was detected at both stages of growth[87]. In contrast to *toxA* transcription, *regAB* T1 and T2 transcripts were detected under both conditions and at both stages of growth. The T2 transcript was more prominent than the T1 transcript at late exponential phase of growth[87]. The detection of the *regAB* T1 transcript indicates that expression from the P1 promoter in PAO1 at early stages of growth is different from previous studies that suggested the *regAB* P1 promoter is not expressed in PAO1 due to the nonfunctional *regB* gene[137].

A recent study by Hunt *et al.*[58] examined the effect of *pvdS* on *toxA* and *regAB* expression throughout the growth cycle of the *P. aeruginosa* strain PA103 under aerobic conditions only. The level of ETA activity produced by

a PA103::*pvdS* mutant was 18–25% of that produced when the mutant was complemented with a *pvdS* plasmid[58]. In agreement with the previously reported analysis of the PAO1::*pvdS* mutant, the reduced level of ETA produced by PA103::*pvdS* was still regulated by iron, further supporting the possibility that ETA production is regulated via *pvdS*-dependent and *pvdS*-independent mechanisms[58]. The study also showed that under aerobic conditions, *toxA* expression in PA103 was regulated by *pvdS* through *regAB*[58]. Expression from the *regAB* P2 promoter throughout the growth cycle of PA103::*pvdS*, as monitored using a *regAB–lacZ* chromosomal fusion, was eliminated under iron-sufficient and iron-deficient conditions[58]. As the PA103::*pvdS* strain carrying the *regAB–lacZ* fusion has a nonfunctional *regAB* locus and, since *regB* is required for P1 expression, it was inferred that *regAB* expression in this strain was directed solely through the P2 promoter[58]. In support of these findings, a sequence that carries eight of nine of the conserved nucleotides within the PvdS IS box binding site was detected between −1 and +8 of the transcriptional start site of T2[58]. This suggests that PvdS directly regulates the *regAB* P2 promoter. Another potential IS box was identified within the *toxA* upstream region raising the possibility that PvdS may also regulate *toxA* expression directly[58]. However, this possibility was discounted by Hunt *et al.*[58] who examined the effect of a *pvdS* plasmid on ETA production in a PA103 strain carrying intact *toxA* but nonfunctional *regAB*. The defect of this mutant in ETA synthesis was not complemented by the presence of the *pvdS* plasmid. Thus, the significance of the potential IS box within the *toxA* upstream region is unknown. Based on additional analysis, Hunt *et al.*[58] suggested that PvdS is not essential for, but may modulate, the expression from the *regAB* P1 promoter. Since the P1 promoter lacks the putative IS box, such modulation may occur indirectly, possibly through *ptxR* (see below).

Recent studies by Lamont *et al.* and Beare *et al.* clearly showed that the production of ETA, pyoverdine, and the PrpL protease by *P. aeruginosa* is regulated by pyoverdine[6, 66]. Similar to the *P. aeruginosa* autoinducers, the pyoverdine molecule functions as a sensor and enhances the production of these three virulence factors through a system that involves the pyoverdine outer membrane protein receptor FpvA, a transmembrane anti-sigma factor FpvR, and PvdS[6, 66]. The amount of ETA produced by a pyoverdine mutant of PAO1 was considerably lower than that produced by PAO1[66]. This defect was eliminated upon the addition of purified pyoverdine to the growth medium[66]. Similarly, the defect in ETA production by PAO1 in which FpvR was overproduced was eliminated by the addition of purified pyoverdine[66].

3.3.3. fur

The negative regulation of *toxA* and *regAB* expression by iron suggested that this regulation is accomplished through a negative regulator that represses

regAB expression in the presence of iron. One possible candidate for such a regulator is Fur[4, 85, 128]. Fur, originally described in *E. coli*[4, 85], is an iron-responsive DNA binding repressor of the siderophore genes in different bacteria[7, 12, 71, 95]. A study by Prince *et al.* provided the earliest indication that *P. aeruginosa* carries a structural and functional homologue of the *E. coli* Fur (EC-Fur)[102]. Immunoblotting experiments revealed the presence of a *P. aeruginosa* protein that cross-reacted with the EC-Fur antibody[102]. In addition, a multicopy plasmid carrying the EC-*fur* gene repressed the production of ETA, *toxA* mRNA, and *regAB* mRNA in the *P. aeruginosa* strain PA103 that was grown in iron-defficient medium under aerobic conditions[102].

Prince *et al.*[101] later isolated the *P. aeruginosa fur* (PA-*fur*) from PA103 through complementation analysis of an *E. coli fur* mutant. The *P. aeruginosa* Fur (PA-Fur) showed extensive amino acid homology to Fur proteins from other gram-negative bacteria but lacks the potential metal binding motif at its carboxy terminus end[101]. Surprisingly, and in contrast to the effect of an EC-*fur* plasmid, a PA-*fur* plasmid did not interfere with the normal regulation of ETA and siderophore production by iron in PA103[101]. One possible explanation for this difference was that PA-Fur may have no affinity for its target sequence when *P. aeruginosa* is grown in iron-deficient medium while the EC-Fur has some affinity for this sequence under the same condition[101]. The exact reason for the difference is not known at this time. To understand further the regulation of the siderophores and ETA by Fur, Prince *et al.*[101] tried to isolate a PA103 *fur* null mutant by the gene replacement technique. However, despite several attempts, no successful *fur* isogenic mutant was constructed[101]. To explain this failure, the authors postulated that *fur* is an essential gene in *P. aeruginosa* and the complete loss of its function is lethal; that is, Fur may regulate other genes besides *toxA* and the siderophore genes[101]. Further analysis by Ochsner *et al.*[89] proved the validity of this assumption. Using a specific selection procedure (Genomic Selex) to enrich for *P. aeruginosa* chromosomal fragments to which purified Fur specifically binds, more than thirty genes whose expression is regulated by iron through Fur were identified[89, 128]. These genes include the siderophore receptor and biosynthesis genes, the heme uptake genes, iron-acquisition genes, *pvdS*, and genes that code for metabolic and detoxifying enzymes among others[89, 128].

As an alternative to the gene replacement approach, Prince *et al.* utilized the manganese selection procedure to isolate a PA103 Fur-deficient mutant[101]. Analysis of several manganese-resistant mutants revealed that one strain, PA103M, produced the pyoverdine and pyochelin siderophores constitutively[101]. In addition, ETA production by PA103M was iron-insensitive. PA103M produced altered Fur protein that migrated faster than the wild-type Fur by SDS-PAGE[101].

Besides confirming the negative regulation of ETA and the siderophores by Fur, the study by Prince *et al.*[101] provided two intriguing findings. First, while PA103M was affected in both ETA and siderophore production, other

manganese-resistant mutants were affected in siderophore production only[101]. It is possible that the specific structural changes introduced into Fur by each mutation affected the ability of the protein to regulate the siderophores, ETA, or both[101]. All mutants produced cross reactive Fur proteins that migrated faster than the PA103 Fur on SDS-PAGE. However, Fur from PA103M, in which ETA and siderophore production were deregulated, did not migrate as fast as Fur from other mutants in which siderophore production only was deregulated[101]. The phenotype of deregulation in siderophore production only was also reported among several manganese-resistant mutants of PAO1[5]. This observation provided the first hint that ETA production by *P. aeruginosa* may be regulated by a Fur-independent mechanism. Second, the PA-*fur* plasmid complemented the defect of PA103M in siderophore production only, yet a deletion derivative of this plasmid complemented the defect in both siderophore and ETA production[101]. Whether other *P. aeruginosa* genes adjacent to *fur* interfere with the effect of Fur on ETA production is not known[101]. Additional in vitro analysis using purified Fur shed more light on the regulation of ETA by Fur[88]. Fur protein purified from *P. aeruginosa* specifically bound to a synthetic DNA fragment carrying the perfect Fur box (GATAATGATAATCATTATC)[88]. The purified Fur also specifically bound to the promoter regions of the pyochelin regulator *pchR* and to *pvdS*, both of which contain the Fur binding box[88]. However, purified Fur did not bind to the promoter regions of either *toxA* or *regAB*[88]. The failure of Fur to bind to *toxA* or *regAB* promoters suggested that Fur regulates *toxA* expression indirectly, possibly through *pvdS*, which is required for *toxA* and *regAB* expression[88].

Barton *et al.* conducted a more in-depth analysis of Fur regulation of ETA by isolation and characterization of several additional manganese-resistant mutants[5]. These mutants were isolated from PAO1; two (A2 and A4) carried point mutations that changed the His-86 residue of Fur, which is located within the conserved His–Asp–His region to either Arg-86 or Tyr-86[5]. The third mutant is cold sensitive (CS) and carries a mutation 4 bp 5′ of the Fur ATG codon[5]. The fourth mutant, C6, was isolated from a PAO1 strain that carried a deletion within the *anr* gene, which activates transcription of the anaerobic respiration genes (the deletion within *anr* had no effect on the production of siderophores or ETA)[5]. Under aerobic conditions, siderophore production by the four mutants was constitutive but ETA production was iron-regulated[5]. However, under microaerobic conditions, ETA production by the A2 and A4 mutants was iron-regulated but was constitutive by the CS and C6 mutants[5]. Further analysis confirmed that in the C6 mutant and under microaerobic conditions, the accumulation of *toxA* and *regAB* mRNA was constitutive with respect to iron[5]. DNA/gel shift experiments showed that higher concentrations of purified Fur proteins from these mutants were required to produce specific gel-shift bands with the *pvdS* promoter[5].

This study reinforced the conclusion that Fur negatively regulates *toxA* and *regAB* expression indirectly by repressing *pvdS* expression. It also clearly indicated that, unlike siderophore regulation, *toxA* regulation by Fur occurs under microaerobic conditions only[5]. Whether this regulation is specific to the PAO1 strain (the PA103-*fur* mutant previously described by Prince *et al.* produced ETA constitutively under aerobic conditions[101]) is yet to be determined. One possible explanation for the constitutive production of ETA by C6 under microaerobic conditions only is that the growth of C6 under these conditions induced a conformational change in Fur that interfered with its binding to the *pvdS* promoter[88]. According to this assumption, the pyoverdine siderophore, which is also regulated by *pvdS*, would be constitutively produced by C6 under microaerobic conditions but iron-regulated under aerobic conditions. Instead, experimental evidence showed that pyoverdine siderophore production by C6 was constitutive under both conditions. A more likely scenario is that under aerobic conditions, *toxA* expression is repressed by iron (at least in PAO1) by a repressor other than Fur[88]. A potential candidate for such a repressor is a protein encoded by the *P. aeruginosa* locus np22[133]. The amino acid sequence of the predicted protein shows homology to other Fur proteins including the PA-Fur[133].

3.3.4. ptxR

ETA production is a complicated process and is likely to involve several genes besides those described above. During complementation analysis of an ETA-secretion deficient mutant, Hamood *et al.*[46] isolated a *P. aeruginosa* PAO1 chromosomal fragment that carried a potential ETA secretion gene. However, further analysis revealed that the gene carried on the fragment enhances ETA synthesis rather than secretion[46]. The presence of a plasmid containing the cloned fragment in either PAO1 or PA103 enhanced ETA production by 4–5 fold[46]. Both subcloning and nucleotide sequence analyses localized the effect on ETA synthesis to a smaller DNA fragment that carried an intact ORF (*ptxR*, for *P*seudomonas *tox*A regulator[46]). *ptxR* encodes a 34-kDa protein (PtxR) that belongs to the LysR family of transcriptional activators[46]. The highest degree of similarity between PtxR and different proteins of the LysR family was localized to the amino terminus region, which contains a helix–turn–helix motif for DNA binding[46]. Despite this high degree of similarity, PtxR does not share other features of LysR proteins. For example, while the genes for most LysR activators are divergently transcribed from their target genes, *ptxR* is distantly located from two of its potential target genes (*toxA* and *regAB*)[46]. Another *ptxR* target, the *pvc* operon that codes for the pyoverdine chromophore, is located 3′ of *ptxR* instead of being divergently transcribed from it[115]. In addition, the gene that is located 5′ of *ptxR* and that is divergently transcribed from it codes for another *toxA* regulator, *ptxS*, which modulates the effect of PtxR[20]. Secondary structure analysis of many LysR proteins showed the presence of functional domains for

DNA interaction at both the amino and carboxy terminus regions and a co-inducer recognition/response region[46]. At this time, it is not known if PtxR binding requires the carboxy terminus region. In addition, neither a co-inducer response region nor the PtxR co-inducer has been identified.

Transcriptional studies, including the analysis of *toxA–lacZ* fusions and the accumulation of *toxA* and *regAB* mRNA, revealed PtxR enhances both *toxA* and *regAB* transcription[21, 129]. However, the presence of a *ptxR* plasmid within the *regA* mutant PA103-29 did not complement its defect in ETA production[46]. These results suggested that PtxR regulates *toxA* expression through *regAB*. Unlike *regAB, ptxR* does not seem to be essential for ETA production. In comparison with its parent strain, the level of ETA produced by the PAO1-*ptxR* isogenic mutant was reduced but not eliminated[46]. The *ptxR* mutant also differs from several other *toxA* regulatory mutant strains in the instability of its phenotype. Upon continuous subculturing, the phenotype of the *ptxR* mutant became similar to that of its parent strain with respect to ETA production even though the mutant still carries the *ptxR* deletion within its chromosome[46]. At this time, it is not known if another *P. aeruginosa* LysR regulator compensates for the defect of the *ptxR* mutant and restores ETA production.

Hamood *et al.* also described a PA103-*ptxR* mutant that was phenotypically stable; that is, the reduction in ETA persisted despite continuous subculturing[46]. This mutant together with *regAB* P1 or P2 transcriptional fusion plasmids were utilized to determine if PtxR regulates the expression from *regAB* P1 or P2 promoter[46]. The level of *regAB* P2 expression was similar in both PA103 and PA103-*ptxR* strains (data not shown). However, the level of *regAB* P1 expression in PA103-*ptxR* was considerably reduced at both early and late stages of growth, suggesting that *ptxR* regulates *regAB* through P1 promoter (Table 2). This suggestion was supported further by the identification of a potential LysR binding site within the *regAB* P1 promoter region[46]. It would be interesting to compare the level of *regAB* P1 expression throughout the growth cycle of PA103 and PA103::*ptxR*.

Table 2. Expression of CAT activity from the *regA* P1 promoter in PA103 and PA103-*ptxR*.

Strain	Chloramphenicol acetyltransferase (CAT) activity (mU/μg)[a]	
	4 hr	8 hr
PA103/pP11[b]	103.5	62.0
PA103-*ptxR*/pP11	0.6	7.1

[a]Cells were grown in TSB-DC and harvested at 4 and 8 hr of growth as the P1 promoter is induced early in the growth cycle of *P. aeruginosa*[40]. The level of CAT activity was determined as previously described[46]. There was no major difference between the growth rates of the strains.
[b]Plasmid carrying the *regA* P1 promoter[117].
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Results of different studies have established that *toxA* and *regAB* expression is positively regulated by PvdS and negatively regulated by iron-activated Fur through PvdS[5, 87, 88, 101]. Thus, with the identification of *ptxR*, it was necessary to examine its place within the complicated circuit. Initial RNase protection analysis revealed that under aerobic conditions, PAO1 produces two separate transcripts; a large one (T1) and a smaller one (T2), suggesting that *ptxR* is transcribed from two promoters[129]. The T1 transcript was constitutively produced in iron-sufficient and iron-deficient media throughout the growth cycle of PAO1; whereas the T2 transcript was produced in iron-deficient medium only and at late logarithmic to early stationary phase of growth, indicating negative regulation by iron[129]. This is somewhat similar to the pattern of *regAB* transcription in PA103, but it differs from it in that the *ptxR* T1 transcript was detected throughout the growth cycle and not at early stages of growth only as is the *regAB* T1 transcript[129].

3.3.4.1. *ptxR* and Fur. To determine if iron regulates *ptxR* transcription through Fur, the production of T1 and T2 transcripts by the PAO1 *fur* mutant C6, in which *toxA* expression is constitutive under microaerobic conditions only, was examined[129]. The production of *ptxR* T1 transcript by C6 and its parent strain was constitutive under both aerobic and microaerobic conditions[129]. T2 production, on the other hand, was iron-regulated in both strains under aerobic conditions but was constitutive in C6 only under microaerobic conditions[129]. Thus, at least part of *ptxR* expression is negatively regulated by iron through Fur under microaerobic conditions only. Similar to *toxA* and *regAB, ptxR* does not appear to be directly regulated by Fur. The *ptxR* upstream region contains two sequences that carry 11 of the 19 conserved nucleotides within the Fur-binding box[129]. However, DNA/gel shift experiments failed to demonstrate specific binding between purified Fur (even at 1 μM concentration) and the *ptxR* upstream region[129].

3.3.4.2. *ptxR* and PvdS. If iron represses expression from the *ptxR* P2 promoter under aerobic conditions through a Fur-independent mechanism, would this mechanism involve PvdS? This possibility was examined by comparing *ptxR* expression under aerobic conditions between PAO1 and its *pvdS* isogenic mutant[129]. Both PAO1 and PAO1-*pvdS* produced similar levels of the *ptxR* T1 transcript in iron-sufficient and iron-deficient media[129]. The level of *ptxR* T2 transcript produced by PAO1 was reduced in the presence of iron[129]. However, the level of *ptxR* T2 transcript produced by PAO1-*pvdS* was reduced from its parent strain but was not affected by iron (constitutive)[129]. This differs from the regulation of *toxA* and *regAB* expression by *pvdS* as neither *toxA* nor *regAB* mRNA was detected in the PAO1-*pvdS* mutant; and indicates that *pvdS* is not essential for *ptxR* expression. However, results of recent studies strongly

suggest that PvdS directly regulates *ptxR* expression by binding to the *ptxR* upstream region[142]. Wilson *et al*. have recently shown that sequences containing most of the conserved nucleotides within the PvdS IS box exist within the upstream region of several pyoverdine synthesis genes (*pvdS, pvdD, pvdE*, and *pvdF*) and *ptxR*[142]. In addition, preliminary DNA/gel shift analysis suggested that partially purified PvdS specifically binds to the 155-bp fragment within the *ptxR* upstream region that carries the IS box[51]. Whether PvdS enhances the binding of the core RNA polymerase to the *ptxR* promoter region as has been demonstrated with the *pvdD* promoter is yet to be determined. It is possible that the apparent partial regulation of *ptxR* by PvdS is due to direct but less efficient binding of PvdS to the *ptxR* upstream region in comparison with the its binding to the pyoverdine gene promoters. Based on these results, it appears that depending on the oxygen level, *ptxR* expression from the P2 promoter is regulated by two mechanisms; a Fur-dependent one under microaerobic conditions and a Fur-independent one in aerobic conditions[51, 129, 142].

3.3.4.3. *ptxR* and *regAB*. Thus far, results suggested that PtxR positively regulates the expression from the *regAB* P1 promoter. It was still unknown, however, if PtxR had any effect on either the expression from the *regAB* P2 promoter or the typical biphasic pattern of *toxA* and *regAB* expression. To address these questions, Colmer and Hamood examined the effect of PtxR on ETA production as well as the accumulation of *toxA* and *regAB* mRNA throughout the growth cycle of PAO1 under aerobic conditions only (the biphasic pattern of *toxA* and *regAB* expression was described in aerobically grown *P. aeruginosa*)[21]. In order to avoid any regulatory problems that may be caused by the presence of multiple copies of *ptxR*, these studies were conducted using the PAO1 strain PAO1-XR that carries two copies of *ptxR* on its chromosome[21]. PAO1-XR was constructed by the integration of a ColEI-*ptxR* plasmid into the PAO1 chromosome through homologous recombination in a single crossover event[21]. In iron-deficient medium, and in comparison with PAO1, PAO1-XR produced significantly higher levels of ETA beginning at an early stage of growth (6-hr time-point) and continuing through the growth cycle to late stationary phase[21]. A similar increase was detected in the level of accumulated *toxA* and *regAB* mRNA[21]. In iron-sufficient medium, neither ETA, *toxA* mRNA, nor *regAB* mRNA was detected at any time point throughout the growth cycle of PAO1 or PAO1-XR[21]. This confirms previous studies that showed even multiple copies of *ptxR* did not interfere with the negative regulation of *toxA* expression by iron. The significant increase in *toxA* and *regAB* mRNA at late stages of growth of PAO1-XR suggests that the expression from the *regAB* P2 promoter is enhanced. However, changes in the level of accumulated mRNA reflect the effect on both the stability of the mRNA and the rate of *regAB* transcription. Therefore, examining the expression of *regAB*

P1 and P2 promoters using transcriptional fusion systems throughout the growth cycle of PAO1 and PAO1-XR will be necessary to confirm that PtxR regulates the *regAB* P2 promoter.

Despite several studies, the exact mechanism through which *ptxR* enhances *toxA* and *regAB* expression is still unknown. Similar to other LysR-type proteins, PtxR contains a DNA binding motif at its amino terminus region and is likely to regulate its target gene by binding directly to its promoter region. To examine such a possibility, Colmer and Hamood overproduced PtxR in *E. coli* using different commercial systems including Affinity/pCAL-n®, FLAG®, and the pBAD/His® system[19, 22]. DNA/gel shift experiments showed that the purified PtxR failed to bind to the *toxA* or *regAB* upstream regions even when *P. aeruginosa* lysate was added to the binding reaction in case PtxR binding requires other cellular factors[19, 22]. This lack of binding may be due to either a conformational change in PtxR during purification or the fact that PtxR regulates *regAB* and *toxA* indirectly by binding to the promoter region of another regulator(s). As an alternative approach, we are currently utilizing microarray analysis to identify the PtxR target gene(s). Comparing the global gene expression between PAO1and PAO1-*ptxR* will help identify genes that are positively or negatively regulated by *ptxR*. Microarray analysis has been recently used by Ochsner *et al.*[90] to identify the *P. aeruginosa* genes that are differentially expressed in response to IS.

The detection of two *ptxR* promoters and their differential regulation with respect to iron suggests that the pattern of *ptxR* expression may be similar to that of *regAB*. Colmer and Hamood examined *ptxR* expression throughout the growth cycle of PAO1 under aerobic conditions[21]. This was done using a *ptxR–lacZ* fusion plasmid that carried the entire *ptxR* upstream region (both P1 and P2 promoters)[21]. In both iron-deficient and iron-sufficient media, *ptxR* expression was detected at an early logarithmic phase of growth and continued throughout the growth cycle[21]. In iron-deficient medium, the pattern of *ptxR* expression resembled that of *regAB* expression in PA103; expression followed a biphasic curve with an initial peak early in the growth cycle followed by a sharp reduction and another peak later in the growth cycle[21]. In iron-sufficient medium, no specific pattern of *ptxR* expression was observed[21]. At several time points, the level of *ptxR* expression in iron-sufficient medium was significantly lower than that in iron-deficient medium[21]. More importantly, unlike *regAB* expression, *ptxR* expression throughout the growth cycle of PAO1 in iron-sufficient medium was reduced but not eliminated[21]. Whether this reduced expression is controlled solely by the P1 promoter or both the P1 and P2 promoters is yet to be determined as these experiments examined the overall expression of *ptxR*. Recently, Harris *et al.*[50] constructed a *ptxR::lacZ* fusion plasmid that carries the P2 promoter region only. Preliminary analysis revealed that *ptxR* expression in this plasmid is negatively regulated by iron when PAO1

is grown to a late stationary phase of growth under aerobic and microaerobic conditions[50].

3.3.4.4. *ptxR* in *P. aeruginosa* Strain PA103. Previous studies proved that a difference in the *regAB* locus between PAO1 and PA103 is partially responsible for the observed variation in the level of ETA produced by these strains. A difference in the effect of *ptxR* on ETA production may also contribute to this variation. To examine this possibility, Carty *et al.*[15] constructed a PA103 strain that carries two copies of *ptxR* on its chromosome (similar to PAO1-XR). Initial experiments showed that strain PA103-2R has unique features with respect to ETA production. Under aerobic conditions, and in iron-deficient medium, the level of ETA produced by PA103-2R was increased 10 fold over that produced by PA103[15]. Such an increase was even more significant than that produced by a multicopy plasmid carrying *ptxR* (5 fold increase)[15]. In addition, ETA production by PA103-2R was partially deregulated with respect to iron[15]. In iron-sufficient medium, a considerable level of ETA was produced by PA103-2R but not by PA103 or PA103 carrying a *ptxR* plasmid[15]. To determine if this effect occurs at the transcriptional level, and to decipher its mechanism, Carty *et al.*[15] compared the levels of accumulated *toxA* and *regAB* mRNA between PA103 and PA103-2R using real-time PCR analysis, which is more sensitive than other methods of RNA analysis. In comparison with PA103, the number of copies of *toxA* mRNA in PA103-2R was significantly increased in iron-deficient and iron-sufficient media (100–140 fold) confirming the results of the ETA analysis[15] (Table 3). However, *regAB* appears to contribute only partially to this phenomenon as the increase in the number of copies of *regAB* mRNA was only 2–3 fold[15] (Table 3).

The simplest explanation for the PA103-2R phenotype is that the integration of the *ptxR* plasmid into the PA103 chromosome resulted in overexpression of *ptxR*. This possibility was eliminated by the real-time PCR analysis. There was an increase in the number of copies of *ptxR* mRNA in

Table 3. Levels of *toxA* and *regA* mRNA in strains PA103 and PA103-2R determined by real-time PCR.

Strain	*toxA* copy number[a]	*regA* copy number
PA103	1.67×10^{9} [b]	2.07×10^{10}
PA103 Fe[c]	2.73×10^{8}	4.81×10^{8}
PA103-2R	1.61×10^{11}	3.27×10^{10}
PA103-2R Fe	3.86×10^{10}	1.5×10^{9}

[a]The mRNA copy number of each gene is expressed per 10^{10} copies of 16S rRNA.
[b]Each value represents the average of two independent experiments conducted in duplicate.
[c]Fe, iron-supplemented medium.

PA103-2R compared to PA103, but that increase was significantly less than that observed in PA103 carrying a *ptxR* plasmid (multiple copies of *ptxR*)[15]. To determine if the effect is due to DNA changes within the integration region, the integrated *ptxR* plasmid together with the neighboring PA103 chromosomal regions was retrieved from the PA103-2R chromosome[15]. The introduction of the retrieved plasmid into PA103 produced a phenotype similar to that of PA103-2R; that is, ETA production was significantly enhanced and partially deregulated with respect to iron[15]. This clearly indicated that the effect in PA103-2R is produced by a *trans*-acting factor and is not limited to changes within the PA103-2R chromosome. Nucleotide sequence analysis of the retrieved plasmid confirmed that the *ptxR* ORF and its 5′ region were intact[15]. However, part of the region 3′ of *ptxR* was deleted[15]. Results of additional experiments suggested that the phenotype of PA103-2R is due to an increase in PtxR activity rather than PtxR synthesis[15], although the mechanism for this increase in activity is unknown. The possibility that deletions within the region 3′ of *ptxR* interrupted a potential ORF encoding a *ptxR* modulator was also eliminated[15]. Although computer analysis indicated the presence of several small potential ORFs within the region 3′ of *ptxR*, expression experiments in both *E. coli* and *P. aeruginosa* failed to detect a translation product[15].

3.3.4.5. *ptxR* and *pvc*. Initial analysis indicated that besides ETA, *ptxR* positively regulates the production of the *P. aeruginosa* siderophore pyoverdine, specifically the chromophore portion of the molecule[46]. Pyoverdine is composed of a fluorescent chromophore and a short peptide arm[13]. The pyoverdine chromophore, or pseudoverdine, is encoded by the four-gene *pvc* operon, which is located 3′ of *ptxR* and transcribed in the opposite orientation. PtxR positively regulates the expression of the *pvc* operon[115]. Results of several studies suggested that PtxR regulates the expression of *toxA* and *pvc* differently[20, 46, 114, 115]. While multiple copies of *ptxR* did not interfere with the negative regulation of *toxA* expression by iron in PAO1 or PA103, *pvc* expression was deregulated[20, 46]. This phenomenon was observed even when *P. aeruginosa* carried two copies of *ptxR*[21]. One possible explanation for this phenomenon is that PtxR enhances *pvc* transcription more efficiently than *toxA* transcription. An alternative possibility is that *pvc* expression is not as efficiently repressed by iron as *toxA* expression. Although conclusive evidence for either possibility is lacking, results of previous analyses support both possibilities. In support of the first possibility, RNase protection experiments revealed that *pvc* expression was eliminated in a *ptxR* isogenic mutant of PAO1 while *toxA* expression in a similar mutant was only reduced[115]. In support of the second possibility, RNase protection studies showed that in iron-sufficient medium, *pvc* transcription in PAO1 was reduced; *toxA* expression is eliminated under such conditions[115]. Regardless of these possibilities, preliminary studies suggest that

toxA regulation by PtxR does not occur through *pvc*[14]. Even in the presence of a *ptxR* plasmid, the production of the pyoverdine chromophore by a PAO1 strain that carries a deletion within the *pvcD* gene was eliminated[14]. However, ETA production was increased significantly[14].

3.3.5. ptxS

Earlier analysis suggested that the region 5′ of *ptxR* contains a gene that modulates the effect of PtxR on ETA synthesis. During attempts to localize *ptxR*, subcloning and complementation experiments revealed that a deletion of a 2-kb fragment from the region 5′ of *ptxR* significantly enhanced the effect of *ptxR* on ETA synthesis in PAO1[20]. Transcriptional studies, using a *toxA::lacZ* fusion, showed that the region 5′ of *ptxR* interfered with the effect of PtxR on *toxA* transcription[20]. Nucleotide sequence analysis confirmed the presence of an ORF (*ptxS*) that was transcribed from the other strand of DNA and in the opposite orientation of *ptxR*. The *ptxS* gene encodes a 38-kDa protein that belongs to the LacI–GalR family of repressors and contains several potential domains for DNA binding, effector-binding, and dimerization[20]. Although the exact mechanism through which PtxS modulates PtxR function is not known, available evidence suggest that PtxS directly interferes with *ptxR* transcription. Transcriptional studies using *ptxR–lacZ* fusion plasmids indicated that deleting the region that carries intact *ptxS* enhanced the level of *ptxR* expression significantly[20]. Despite these results, partially purified PtxS did not bind to the *ptxR* upstream region. PtxS autoregulates its synthesis by binding specifically to a 14-bp palindromic sequence within the *ptxS* upstream region[20, 120, 121]. This binding, however, may still regulate *ptxR* expression as the *ptxR/ptxS* upstream regions overlap. Nested deletion analysis, using *ptxS–lacZ* fusions, confirmed that deletion of the region that overlaps with the *ptxR* promoters reduced the level of *ptxS* expression significantly[45, 121]. The relationship between *ptxR* and *ptxS* was complicated further by the discovery that *ptxS* is the first gene of the five-gene *kgu* operon that is involved in the utilization of 2-ketogluconate by *P. aeruginosa*[120]. With the exception of *ptxS*, all other genes of the operon encode enzymes involved in 2-ketogluconate utilization[120]. The expression of the *kgu* operon appears to be regulated by PtxS in response to the level of 2-ketogluconate (the effector molecule)[120]. In vitro binding of PtxS to its target sequence was inhibited in the presence of 10 mM 2-ketogluconate[120]. Interrogation of the PAO1 genomic sequence revealed the presence of an identical PtxS binding site between *ptxS* and *kguE*, the second gene in the *kgu* operon[120]. PtxS specifically binds to this site and its binding is inhibited by 2-ketogluconate[120]. If PtxS binds as a dimer to the two sites, it would loop out the entire *ptxS* gene. More importantly, beside the direct effect of PtxS on *ptxR* expression, these results suggest that one of the metabolic pathways—the gluconate utilization pathway—may regulate ETA synthesis in *P. aeruginosa*.

In addition to identifying potential regulatory sequences within the *ptxR/ptxS* intergenic region through nested deletion analysis, we have used different portions of the intergenic region in DNA binding experiments to identify putative regulatory proteins. Using this approach, Swanson *et al.*[119] showed that the lysate of PAO1 contains several proteins that specifically bind to different segments of the *ptxR/ptxS* intergenic region. One of these proteins is PtxS, which binds to the *ptxS* upstream region, as described above[119, 121]. Additional proteins bound to a 150 bp fragment within the *ptxR/ptxS* intergenic region. To partially purify these proteins, Westfall *et al.*[136] first concentrated the proteins from several one-liter cultures of PAO1 and then enriched for DNA binding proteins among them using heparin-sepharose columns. One fraction from the heparin-sepharose column that produced specific binding to the 150-bp fragment contained two proteins, one 15 kDa and the other 9 kDa[136]. The first 20 amino acids of each protein were determined. Analysis of the PAO1 genome using these sequences revealed that the 9-kDa protein is the *P. aeruginosa* HU protein while the 15-kDa protein is a homologue of MvaT[136]. MvaT is a transcriptional activator that regulates the expression of the *mvaAB* operon, which catalyzes the initial stage of the mevalonate catabolism in *Pseudomonas mevalonii*[106]. Diggle *et al.*[28] have recently described the *P. aeruginosa* MvaT as a global regulator of virulence factors. Pyocyanin production, as well as the expression of *lecA*, which codes for the *P. aeruginosa* PA-IL lectin, were increased in PAO1 that carried a specific *mvaT* deletion[28]. The mutation also affected the production of the elastases LasB and LasA and the two autoinducers, 3O-C_{12}-HSL and C_4-HSL[28]. Whether MvaT regulates the expression of *ptxR, ptxS*, or both is yet to be determined.

3.3.6. vfr

The presence of more than one *toxA* regulator was suggested as early as 1980. Gray *et al.*[42] described two nitrosoguanidine-induced mutants of PAO1 that produced low levels of ETA. The mutations in those two strains (identified as *toxA*-1 and *toxA*-2) were mapped to two separate locations on the PAO1 chromosome[42]. Using a similar approach, Ohman *et al.*[93] isolated several ETA-deficient mutants of PA103. In comparison with their parent strain, the mutants lacked several extracellular proteins and were protease-deficient[93]. One mutant, PA103-29, carried a mutation within *regAB*[138]. However, the other four mutants carried no defect in either *toxA* or *regAB*; that is, their defect in ETA production was not complemented by either a *toxA* or a *regAB* plasmid[134]. West *et al.* compared the expression of *toxA* and *regAB* at different stages of growth of the mutants[134]. While the mutants differed from each other in *toxA* expression, they were similar in *regAB* expression[134]. In one mutant, no *toxA* expression was detected; whereas in two of the other mutants, *toxA* was expressed at a reduced level[134]. Interestingly, this reduced level of *toxA* expression was constitutive with

respect to iron[134]. Analysis of *regAB* expression revealed that, in all mutants, the expression from the *regAB* P2 promoter was normal whereas the expression from the *regAB* P1 promoter was reduced[134]. Based on these analyses, the four PA103 ETA-deficient mutants appeared to carry defects in a gene(s) that regulates the expression of *toxA* and *regAB* P1[134]. The reduction in the expression of *toxA* but not *regAB* P2 at later stages of growth suggests the presence of a gene that positively regulates *toxA* expression directly and independently of *regAB* as *toxA* expression at late stages of growth requires the *regAB* P2 promoter. Alternatively, this potential *toxA* regulator may activate the transcription of both *toxA* and *regAB*. Through complementation and subcloning analyses, West *et al.*[135] isolated the *P. aeruginosa vfr*, which complemented the defect of the mutants in the production of ETA and proteases. In addition, a *vfr* plasmid restored the level of *regAB* P1 expression in the mutants to that of their parent strain[135]. The *vfr* gene codes for a 28-kDa protein (Vfr) that is 90% homologous and 67% identical to the *E. coli* cyclic AMP receptor protein, CRP[135]. In addition, Vfr binds to the *E. coli lac* promoter, a reaction that requires cAMP[135]. Similar to the *E. coli* CRP, Vfr is a global regulator of gene expression in *P. aeruginosa*. Two-dimensional polyacrylamide gel electrophoresis revealed that a PAO1-*vfr* null mutant differs (positively or negatively) from its parent strain in the production of 60 proteins[118]. Part of the global regulatory nature of Vfr was demonstrated through its effect on the *P. aeruginosa las* quorum sensing system[2]. Vfr specifically binds to the upstream region of the quorum sensing transcriptional regulator *lasR* and enhances its transcription[2]. Vfr appears to regulate the expression of *toxA* and *regAB* directly. Purified Vfr binds to the upstream region of *toxA* and *regAB*[107]. Besides this direct regulation, Vfr may regulate the expression of *toxA* and *regAB* indirectly through *ptxR*. A putative Vfr binding site was identified within the *ptxR* upstream region[64] and Vfr was shown to specifically bind to the 5′-ACCGTCTGAAGCAGTTCTCATTA-3′ sequence within the *ptxR/ptxS* intergenic region[33]. Furthermore, in comparison with PAO1, *ptxR* expression in the PAO1-*vfr* mutant was reduced[33].

4. MODELS OF *toxA* REGULATION

Based on available results, we propose the following models for the regulation of *toxA* expression in *P. aeruginosa*.

4.1. Low Iron, Aerobic and Microaerobic Conditions

In low iron medium and under both aerobic and microaerobic conditions, *toxA* expression is positively regulated by at least four genes: *pvdS, regAB, ptxR*, and *vfr* (Figure 1A). The main regulation of *toxA* occurs through *pvdS* and *regAB*. The extracytoplasmic factor PvdS enhances expression from the *regAB*

P2 promoter; the increased level of RegA synthesized then enhances *toxA* expression. Regulation of *toxA* expression by *ptxR* is less stringent than that by RegA. Similar to *regAB*, expression from the *ptxR* P2 promoter is positively regulated by PvdS. PtxR enhances *toxA* expression through *regAB*, although the exact mechanism of this regulation is not known. Available evidence suggests that this regulation could be indirect (through an intermediate regulator) or direct through the effect of PtxR on the *regAB* P1 promoter. Under the conditions described above, the expression of the pyoverdine genes is induced; the synthesized pyoverdine molecules may enhance *toxA* expression through a mechanism that involves PvdS, the pyoverdine receptor FpvA, and the transmembrane anti-sigma factor FpvR. Although the regulation of *toxA* expression by Vfr was not extensively analyzed, available evidence suggests that this regulation occurs directly by Vfr binding to the *toxA* upstream region and indirectly by Vfr enhancement of transcription from the *regAB* P1 and *ptxR* P2 promoters.

4.2. High Iron, Aerobic Conditions

In high iron medium and under aerobic conditions, at least in the strain PAO1, a potential iron-dependent regulator other than Fur may negatively regulate *toxA* expression (Figure 1B). This regulator interferes with *pvdS* expression. Consequently, most of the expression from the PvdS-dependent *regAB* P2 and *ptxR* P2 promoters is either eliminated or significantly reduced. As a result, *toxA* expression at late stages of growth of *P. aeruginosa* is eliminated. However, *toxA* expression at early stages of growth still occurs, influenced by the iron-independent *regAB* P1 promoter. The influence of the expression from the iron-independent *ptxR* P1 promoter is not known. The above-described potential regulator may also regulate *toxA* expression to a lesser extent by a PvdS-independent mechanism, the details of which are not known. Another mechanism through which iron represses *toxA* expression is its interference with the expression of the pyoverdine genes and the synthesis of pyoverdine. Thus, the pyoverdine-induced enhancement of *toxA* expression does not occur.

4.3. High Iron, Microaerobic Conditions

In high iron medium and under microaerobic conditions (Figure 1C), the regulation of *toxA* is basically similar to that described in Panel B, except that *pvdS* expression is repressed by iron-activated Fur, which binds to the Fur box within the *pvdS* upstream region. Unlike *toxA*, the pyoverdine genes appear to be negatively regulated by iron-activated Fur under both aerobic and microaerobic conditions.

Recent analyses of *ptxR* suggest the regulation of *toxA* expression is even more complicated. At least in strain PA103, certain changes within the

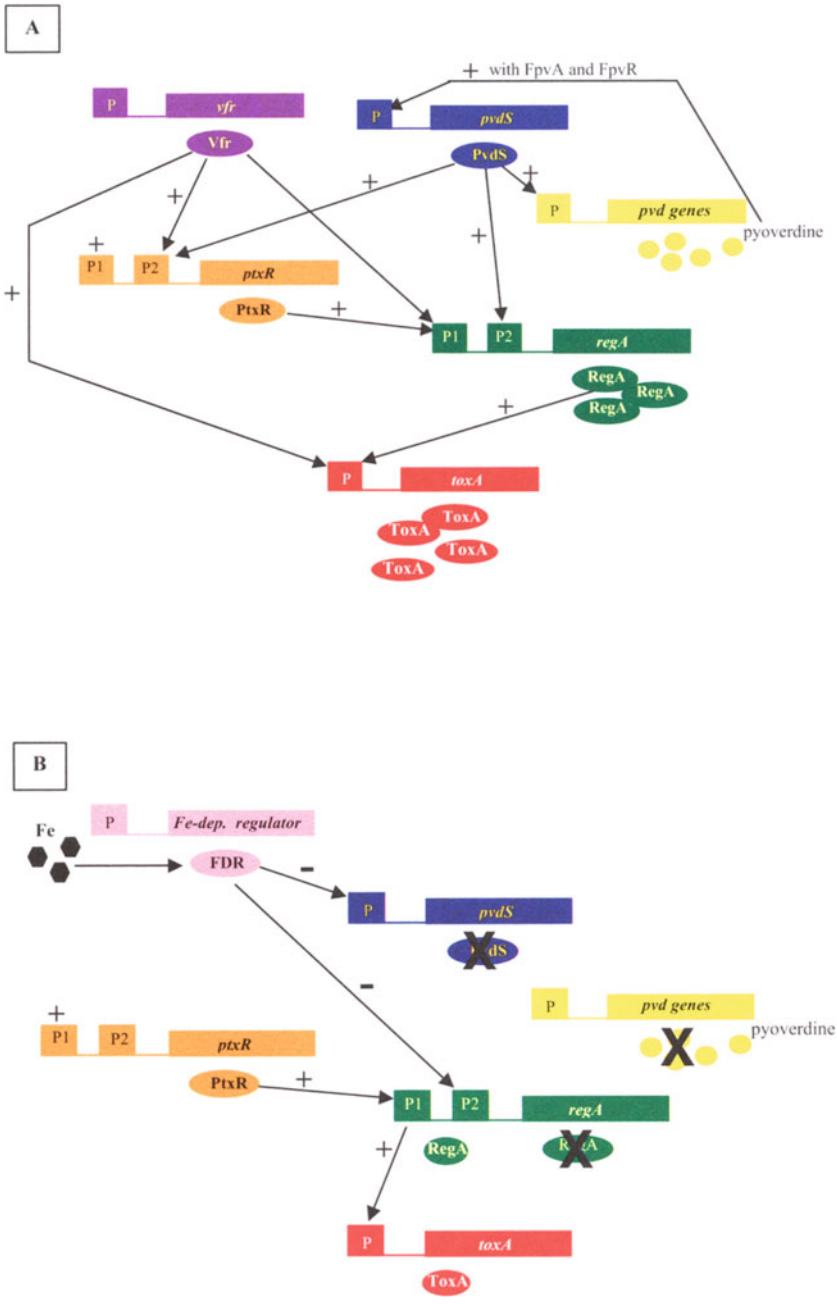

Figure 1. Continued

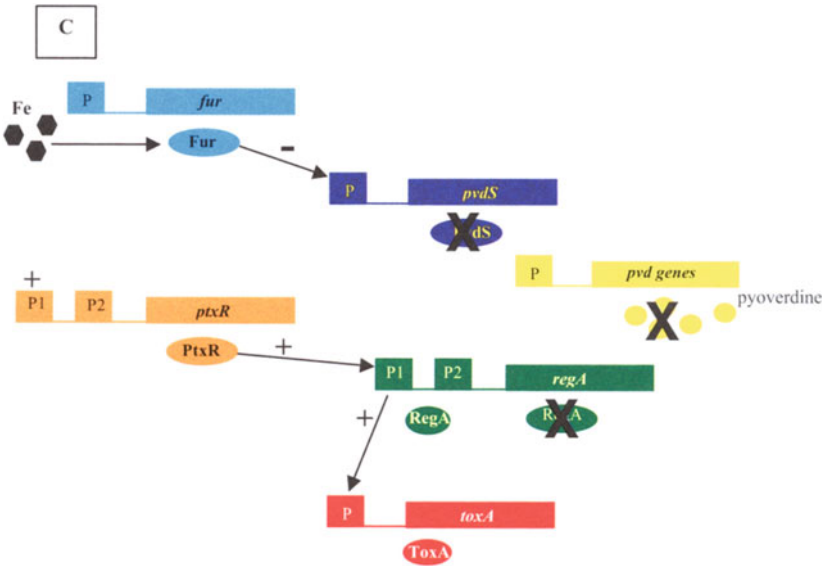

Figure 1. Schematic diagrams of the models of ETA regulation under different conditions. (A) Under low iron, aerobic and microaerobic conditions, *regAB, pvdS, ptxR*, and *vfr* participate in the positive regulation of *toxA* expression at different levels. However, only *regAB* and *pvdS* are essential for *toxA* expression (see text). (B) Under high iron, aerobic conditions, *toxA* expression is negatively regulated by a potential iron-dependent but Fur-unrelated negative regulator. In the presence of iron, the potential regulator interferes with the positive regulation of *toxA* expression by pvdS. It also affects the expression of *regAB* and *ptxR*. (C) Under high iron, microaerobic conditions, *toxA* expression involves the same regulatory genes described in (B), except the iron-dependent negative regulator is Fur.

region 3′ of *ptxR* partially interfere with the regulation of *toxA* expression. This interference appears to be unrelated to Fur. In addition, the *ptxR* upstream region contains potential binding sites for several regulators including the anaerobic response regulator, Anr. Whether *ptxR* is truly regulated by Anr and the significance of this regulation to *toxA* expression is not known.

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15

LESSONS FROM THE *ami* OPERON

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1. EARLY INVESTIGATIONS

In 1958 Patricia Clarke FRS began work at UCL on the amidase system of *Pseudomonas aeruginosa* PAC1. At this time the majority of microbial geneticists were involved with the gut organism *Escherichia coli*. It was clear however that the Pseudomonads were a catabolically much more versatile group of microorganisms with complex degradative pathways involving inducible enzymes. The reasons for choosing the amidase system were: first, that the major pathway for terminal respiration in *P. aeruginosa* was the tricarboxylic acid cycle and thus breakdown products from acetamide hydrolysis could be rapidly metabolised; second, that aliphatic amides could serve as both carbon and nitrogen growth substrates; and finally, a number of aliphatic amides were available which would allow the type of investigation pursued by Monod and colleagues with lactose analogues studying the *lac* operon to be undertaken. The initial studies showed that the substrate and inducer profiles of the amidase system were distinct such that acetamide, propionamide, lactamide and N-acetylacetamide were the best enzyme inducers and formamide, butyramide and phenylacetamide the most active amide analogue co-repressors of the system[20]. With respect to the enzyme substrate specificity, acetamide, propionamide and glycollamide were good substrates, formamide and lactamide poor substrates and trace activity was seen using butyramide

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and N-acetylacetamide. The enzyme was shown to have hydrolase activity, transferase activity in the presence of hydroxylamine and limited esterase activity. Experiments with cells in liquid culture showed enzyme induction and repression as described above and additionally showed that enzyme synthesis was subject to catabolite repression with carbon sources that supported high growth rates causing the greatest repression[6].

1.1. Mutant Isolation and Investigation

The first mutants isolated were those that showed constitutive amidase synthesis. These fell into a number of different classes: semi-constitutive but subject to further induction (PAC109); high constitutive and still subject to butyramide repression (PAC111); high constitutive and subject to N-acetylacetamide repression (PAC124); high constitutive and not subject to amide repression (PAC142)[7, 9]. The initial selections were carried out using succinate/formamide plates and this selection also threw up a number of novel formamide inducible mutants (including PAC153). Amidase negative, *amiE* gene mutants were also isolated and identified by the production of cross-reacting material, and transduction analysis showed very close linkage between the *amiE* gene mutations and the *amiR* regulator mutations[7]. Using these initial mutants as the starting points a number of strains were isolated/constructed able to utilise novel amides as growth substrates (see later).

1.2. Regulatory Studies

Because of the dominance of the *lac* operon in microbial genetics at this time it was initially assumed that amidase operon expression would be regulated by a negative control system. However, repeated attempts to isolate temperature sensitive regulatory mutants that were inducible at low temperature and constitutive at high temperature failed. From the constitutive parent PAC111 and the formamide inducible mutant PAC153 temperature sensitive mutants were isolated that showed constitutive amidase synthesis at low temperature and were amidase negative at high temperature – a phenotype expected for a positively regulated system. The mutations in these strains were predicted to be located in *amiR* since the amidase enzymes produced at low temperature were as thermostable as the wild type[17]. The positive control model for AmiR regulation of amidase expression was thus accepted. The evidence for additional negative regulation (by AmiC) was also here as well since in all of these mutants at low temperature expression was constitutive rather than inducible.

1.3. The Amidase Enzyme

Early studies gave an M_R value of 200 kDa for the native wild-type A enzyme and 35 kDa for the monomeric form indicating a homo-hexameric structure[10]. DNA sequencing and amino acid sequencing later showed a primary structure of 346 residues. Amongst the early mutants isolated was an enhanced substrate-specificity butyramide utilising amidase – the B enzyme[8]. This change allowed growth of the constitutive mutant PAC351 on butyramide despite being sensitive to butyramide repression since the B amidase was able to hydrolyse butyramide faster than it caused repression of enzyme synthesis. However, the B6 mutation did not allow growth on valeramide as a carbon source and PAC351 was used for selection of further mutants able to so do. Some of this family of mutant enzymes were sufficiently changed from the wild type as to be unable to utilise acetamide and most appeared to be unstable. A number of mutant enzymes able to degrade phenylacetamide for use as a nitrogen source were also isolated. The parental strains for these mutant isolations included the original B enzyme producer, some of the valeramide utilising enzyme producers, and two different constitutive parents both resistant to butyramide repression. Thus enzymes able to degrade phenylacetamide have been "evolved" from the original A amidase via four different routes (Figure 1).

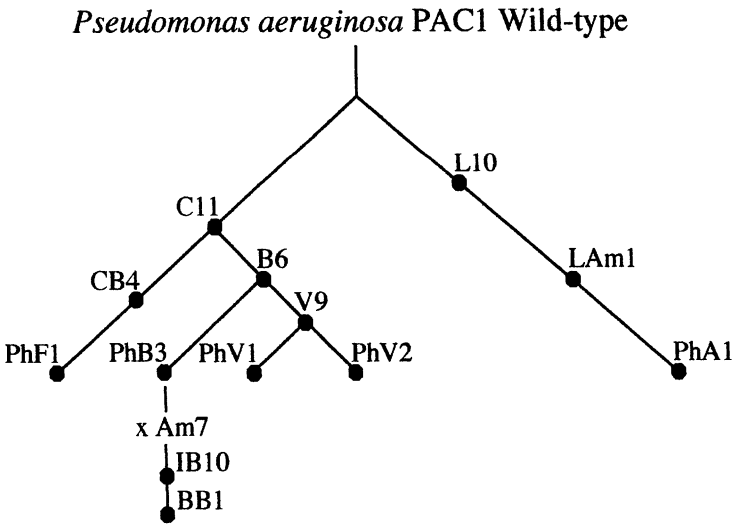

Figure 1. Family tree of amidase mutants with changed regulatory phenotypes and altered substrate specificities. C11, CB4, BB1 and L10 are regulatory mutants. Am7 and LAm1 are amidase-negative mutants obtained by negative selection. Other mutants produce amidases with novel substrate specificities that enable growth on butyramide (B6, BB1), valeramide (V9) and phenylacetamide (PhF1, PhB3, PhV1, PhV2, PhA1). Details are provided in Table 1.

Table 1. *P. aeruginosa* amidase mutants with changed regulatory phenotypes and altered enzyme properties.

Strain	Mutant number	Regulatory phenotype	Characteristics
PAC1	WT	Acetamide inducible	WT A amidase produced[20]
PAC111	C11	High constitutive	Sensitive to butyramide repression[7]
PAC128	CB4	High constitutive	Resistant to butyramide repression[9]
PAC392	PhF1	High constitutive	Phenylacetamide hydrolysis[4]
PAC351	B6	High constitutive	B amidase produced[8]
PAC377	PhB3	High constitutive	Phenylacetamide hydrolysis[4]
PAC307	Am7	Acetamide inducible	Amidase negative[7]
PAC398	IB10	Acetamide inducible	B amidase produced[3]
PAC181	BB1	Butyramide inducible	B amidase produced[32]
PAC360	V9	High constitutive	Valeramide hydrolysis[8]
PAC388	PhV1	High constitutive	Phenylacetamide hydrolysis[4]
PAC389	PhV2	High constitutive	Phenylacetamide hydrolysis[4]
PAC142	L10	High constitutive	Resistant to butyramide and catabolite repression[9]
PAC326	LAm1	High constitutive	Amidase negative[4]
PAC391	PhA1	High constitutive	Phenylacetamide hydrolysis[4]

2. RECOMBINANT DNA STUDIES

In 1977 a chromosomal gene library was constructed using DNA from a high expressing, catabolite repression resistant, amidase constitutive mutant strain PAC433[30] — expected to increase the chances of expression in the heterologous *E. coli* host, and *Eco*RI and *Hin*dIII bacteriophage lambda replacement vectors. Only the *Hin*dIII library yielded a single amidase positive recombinant carrying an ≈9 kbp insert with the loss of one of the *Hin*dIII insertion target sites — the chromosomal *Hin*dIII *ami* fragment is 21.5 kbp in length[15,28]. Restriction enzyme analysis and the construction and analysis of phage deletion mutants located the amidase gene(s) to a 5.3 kbp *Hin*dIII/*Sal*GI fragment subsequently sub-cloned into pBR322 forming pJB950.

2.1. The *amiE* Gene

At this time it was only clear that the *amiE* structural gene was present and expressed, and further sub-cloning studies located the gene to within a 2.4 kbp *Hin*dIII/*Kpn*I region. Saturation restriction enzyme mapping the *Hin*dIII/*Kpn*I region then identified the location of *amiE*. Richard Ambler and Tony Auffret in Edinburgh had been working on the determination of the amino acid sequence of amidase — which had been established for a major part of the polypeptide at this time. The limited DNA sequence information available from the mapping and the partial amino acid sequence were compared and

analysed and the location of *amiE* correctly predicted — with the N-terminal end close to the *Hin*dIII target[12].

The cloned gene(s) were then put back into *P. aeruginosa* to measure amidase activity produced by complementation with chromosomally located *amiR* genes. A smaller derivative of pJB950 was made by deletion of downstream *Xho*I fragments and the resultant construct fused to a broad host range RSF1010 derivative. The new plasmid was mobilised into a number of PAC *ami* mutant strains and amidase activity determined. These studies confirmed the positive control model for amidase expression by showing *trans* complementation by AmiR and also confirmed the genotypes of a number of presumed *amiE* mutants including PAC307 and PAC326[14]. The amino acid sequencing of amidase was finally completed when the DNA sequence of the *amiE* gene became available[2, 5]. The amino acid changes found in the butyramide utilising (S7F) and acetanilide utilising (T103I) substrate specificity mutant enzymes were also identified at this time. The *amiE* gene which can be highly expressed shows a heavily biased codon usage in favour of G or C in the third position and only 44 sense codons are used[5]. More recent studies have shown that the *P. aeruginosa* amidase lacks the amidase signature group conserved motif[11] but is instead a member of the nitrilase/cyanide hydratase family along with a number of other amidases[23].

2.1.1. Sequence Homologues of amiE

Current protein sequence analysis has shown that the *P. aeruginosa* amidase belongs to the carbon–nitrogen hydrolase family (pfam00795). As well as aliphatic amidases the family includes: Nitrilases (EC:3.5.5.1), Biotidinases (EC:3.5.1.12) and Beta-ureidopropionases (EC:3.5.1.6). CLUSTALW[31] sequence alignment showed amidases with significant homology to AmiE including the aliphatic amidases of *Geobacillus stearothermophilus, Bradyrhizobium japonicum, Bacillus* sp. BR449, and the aliphatic amidase of *Helicobacter pylori* 26695 (*amiE*) – Table 2. This extensive homology indicates a direct evolutionary relationship. Other amidases such as the enantioselective amidase of *Agrobacterium tumefasciens* detected in the database had significantly lower similarity scores.

2.2. The *amiR* Gene

Initially it was believed that because the *amiE* gene lay at one end of the cloned DNA fragment the *amiR* gene was missing from our constructs — an argument spuriously based on the organisation of the *lac* operon of *E. coli*. However, a comparison of the enzyme activities produced by plasmids in *E. coli* showed that deletion of the *Xho*I fragments downstream of *amiE* in pJB950 reduced the amidase SA from 4.1 to 0.2 and gave us the location of

Table 2. Multiple sequence alignment of seven representative amidases.

```
AAF14257        MRHGDISSSHDTVGVAVVNYKMPRLHTKKEVIENAKNIANMIVGMKQGLPGMDLVIFPEY 60
AAF69000        MRHGDISSSHDTVGIAVVNYKMPRLHTKAEVIENAKKIADMVVGMKQGLPGMDLVVFPEY 60
P11436 AmiE     MRHGDISSSNDTVGVAVVNYKMPRLHTAAEVLDNARKIADMIVGMKQGLPGMDLVVFPEY 60
AC_Q01360       MRHGDISSSNDTVGVAVVNYKMPRLHDRAGVLENARKIADMMIGMKTGLPGMDLVVFPEY 60
NP207092        MRHGDISSSPDTVGVAVVNYKMPRLHTKNEVLENCRNIAKVIGGVKQGLPGLDLIIFPEY 60
NP767613        MLHGDISSSNDTVGVAVVNYKMPRLHTKAEVLDNARKIADMVVGMKVGLPGMDLVIFPEY 60
                * ******* ****:***********    *::*.::**.:: *:* ****:**::****

AAF14257        STMGIMYDRKEMFETATTIPGPETEIFAEACRKANTWGVFSLTGEQHEEHPHKNPYNTLV 120
AAF69000        STMGIMYDQDEMFATAASIPGEETAIFAEACKKADTWGVFSLTGEKHEDHPNKAPYNTLV 120
P11436 AmiE     SLQGIMYDPAEMMETAVAIPGEETEIFSRACRKANVWGVFSLTGERHEEHPRKAPYNTLV 120
AC_Q01360       STQGIMYNEEEMYATAATIPGDETAIFSAACREADTWGIFSITGEQHEDHPNKPPYNTLI 120
NP207092        STHGIMYDRQEMFDTAASVPGEETAIFAEACKKNKVWGVFSLTGEKHEQAK-KNPYNTLI 119
NP767613        STQGIMYDSKEMYETASAVPGEETAIFAEACRKAKVWGVFSLTGERHEEHPHKAPYNTLI 120
                *  ****:  **  ** ::** ** **: **:: ..**:**:***:**:   * *****:

AAF14257        LINNKGEIVQKYRKIIPWCPIEGWYPGDTTYVTEGPKGIKISLIICDDGNYPEIWRDCAM 180
AAF69000        LINNKGEIVQKYRKIIPWCPIEGWYPGDTTYVTEGPKGLKISLIVCDDGNYPEIWRDCAM 180
P11436 AmiE     LIDNNGEIVQKYRKIIPWCPIEGWYPGGQTYVSEGPKGMKISLIICDDGNYPEIWRDCAM 180
AC_Q01360       LIDNKGEIVQRYRKILPWCPIEGWYPGDTTYVTEGPKGLKISLIICDDGNYPEIWRDCAM 180
NP207092        LVNDKGEIVQKYRKILPWCPIECWYPGDKTYVVDGPKGLKVSLIICDDGNYPEIWRDCAM 179
NP767613        LMNDKGEIVQKYRKIMPWVPIEGWYPGNCTYVSEGPKGLKVSLIICDDGNYPEIWRDCAM 180
                *::::*****:****:** *** ****. *** :****:*:***:*************

AAF14257        KGAELIVRCQGYMYPAKEQQIMMAKTMAWANNVYVAVANATGFDGVYSYFGHSAIIGFDG 240
AAF69000        KGAELIVRCQGYMYPAKEQQIMMAKAMAWANNTYVAVANATGFDGVYSYFGHSAIIGFDG 240
P11436 AmiE     KGAELIVRCQGYMYPAKDQQVMMAKAMAWANNCYVAVANAAGFDGVYSYFGHSAIIGFDG 240
AC_Q01360       KGAELIVRCQGYMYPAKDQQVMMSKAMAWANNCYVAVANAAGFDGVYSYFGHSAIIGFDG 240
NP207092        RGAELIVRCQGYMYPAKEQQIAIVKAMAWANQCYVAVANATGFDGVYSYFGHSSIIGFDG 239
NP767613        KGAELIVRCQGYMYPAKEQQVLISKAMAWANNVYVAVANAAGFDGVYSYFGHSAIIGFDG 240
                :*****************:**: : *:*****: *******:************:******

AAF14257        RTLGECGEEENGIQYAEISLSQIRDFRQNAQSQNHLFKLLHRGYTGIIQSGEGDKGVAEC 300
AAF69000        RTLGECGTEENGIQYAEVSISQIRDFRKNAQSQNHLFKLLHRGYTGLINSGEGDRGVAEC 300
P11436 AmiE     RTLGECGEEEMGIQYAQLSLSQIRDARANDQSQNHLFKILHRGYSGLQASGDGDRGLAEC 300
AC_Q01360       RTLGETGEEEYGIQYAQLSVSAIRDARENDQSQNHIFKLLHRGYSGVHAAGDGDKGVADC 300
NP207092        HTLGECGEEENGLQYAQLSVQQIRDARKYDQSQNQLFKLLHRGYSGVFASGDGDKGVAEC 299
NP767613        RTLGECGEEDYGIQYAQLSKHLIRDARRNGQSQNHLYKLVHRGYTGMINSGESPRGVAAC 300
                :**** * *: *:***::*   *** *   ****:::*::****:*:  :*:. :*:* *

AAF14257        PFDFYRTWVMDAEKARENVEKITRTTIGTAECPIEGIPHEGKEKEASV 348
AAF69000        PFDFYRTWVLDAEKARENVEKITRSTVGTAECPIQGIPNEGKTKEIGV 348
P11436 AmiE     PFEFYRTWVTDAEKARDNVERLTRSTTGVAQCPVGRLPYEGLEKEA-- 346
AC_Q01360       PFEFYKLWVTDAQKAQERVEAITRDTVGVADCRVGNLPVEKTVEA--- 345
NP207092        PFEFYKTWVNDPKKAQENVEKITRPSVGVAACPVGDLPTK-------- 339
NP767613        PYDFYKNWIKDPEGTRDMVEAMTRSTPGTDECPIEGIPNEAAASNY-- 346
                *::**: *: *.: ::: ** :** : *.  * :  :* :
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Multiple sequence alignment of seven representative amidases generated by CLUSTALW[31]. Species corresponding to sequence accession numbers are: *Geobacillus stearothermophilus* (AC; AAF14257); *Bacillus* sp. BR449 (AC; AAF69000); *Rhodococcus erythropolis* (AC; Q01360); *Helicobacter pylori* (AC; NP207092) and *Bradyrhizobium japonica* (NP767613). The *P. aeruginosa* amidase (AmiE AC; P11436) sequence is in grey.

The database search was carried out with the *P. aeruginosa* amidase (AC; P11436) as the query sequence using BLASTP version 2.2.6 algorithm[1] at the NCBI. CLUSTALW[31] version 1.82 was accessed through the European Bioinformatics Institute (EBI) and used for multiple sequence alignments.

amiR — downstream of *amiE*[13]. The construction of broad host range derivates of intact pJB950 gave extremely high constitutive amidase activity in *P. aeruginosa* (SA of greater than 150). The location of *amiR* was defined by analysis of *Bal31* generated deletions in pJB950 and sub-cloning studies, and

the gene was finally located to within a 1.0 kbp *Cla*I/*Xho*I region some 2 kbp downstream of *amiE* and expressed in the same direction. A 1.5 kbp *Xho*I/*Xho*I fragment shown to express *amiR* was sub-cloned into a number of expression vectors for complementation studies in *E. coli* and *P. aeruginosa*. Studies in *P. aeruginosa* confirmed the genotypes of a number of presumed *amiR* mutants including PAC327. In all of these investigations the *amiR* gene fragment used was derived from strain PAC433 that shows constitutive amidase expression, and assays showed constitutive amidase expression — as expected. Despite some evidence for an additional regulatory element we had assumed that the $amiR^{Con}$ gene carried mutations such that AmiR functioned as a positive effector in the absence of inducing ligands. The DNA sequence of the 1001 bp *Cla*I/*Xho*I fragment was determined and codon preference analysis identified the *amiR* gene sequence as a 196 amino acid ORF[21]. As with *amiE* only a limited number of codons were used (41 of the 61 sense codons) with a strong bias towards codons with G or C in the third position. Homology searches at that time showed no related sequences in the nucleotide or protein databases.

2.3. The Control Region

At the same time Nick Lowe determined the DNA sequence of the operon leader region[16]. This showed a consensus *E. coli* sigma-70 promoter located 150 bp upstream of *amiE*, a leader ORF of 35 amino acids, a Rho-independent transcription terminator next to a Rho-dependent terminator, and the *amiE* RBS followed by the gene (Figure 2). The Rho-independent terminator DNA sequence showed a 16 bp GC rich stem with four-residue loop followed by a run of six T's. Deletion of the DNA (10 bp) between two *Sma*I targets symmetrically arranged in the stem region led to the formation of a plasmid that expressed high constitutive AmiR-independent amidase activity. Thus the anti-termination regulatory mechanism was proposed with AmiR functioning as a transcription anti-termination factor.

2.4. The Wild-Type Genes

Using a similar selection strategy to the initial cloning the wild-type amidase genes were cloned from PAC1 DNA initially into a phage lambda vector by Alison Sparrow and the isogenic 5.3 kbp *Hin*dIII/*Sal*GI fragment from the ami^{+} phage sub-cloned into pBR322 forming plasmid pAS20. Stuart Wilson carried out the subsequent investigations. Comparison of the leader region DNA sequences of PAC1 and PAC433 showed a single base-pair difference between the two within the *amiE* RBS of the PAC433 sequence.

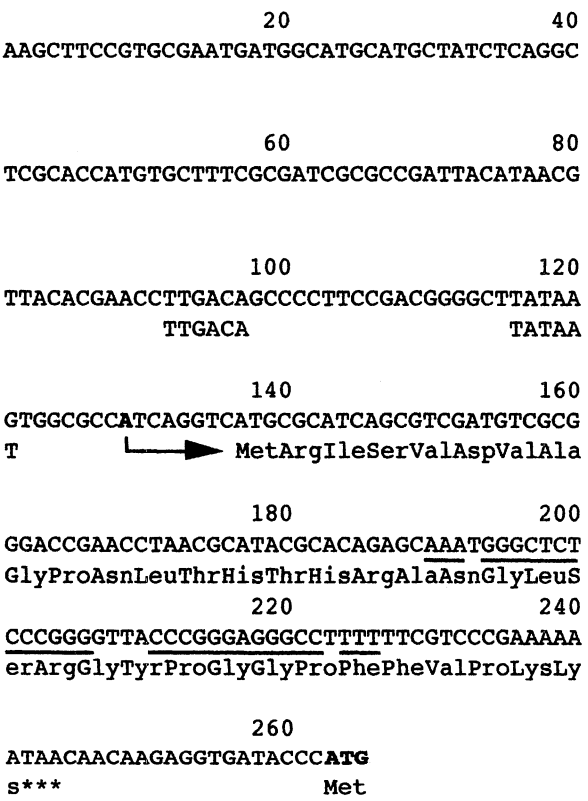

Figure 2. DNA sequence of the amidase operon leader region. The sequence runs from the *Hin*dIII target to the start of the *amiE* gene. The sequence of the *E. coli* consensus sigma-70 promoter is shown below 92–97 (−35 region) and 116–121 (−10 region). The transcription start point[129] is shown in bold, the leader ORF is translated, the dyad symmetry of the Rho-independent transcription terminator is shown by underlining, and the *amiE* start codon is in bold.

This located the "up-promoter" mutation of PAC433 that leads to a 5-fold increase in amidase expression from the mutant. *E. coli* carrying pAS20 shows barely detectable amidase activity and it was thus impossible to determine if expression was inducible or constitutive. However, deletion of the 650 bp *Xho*I fragment downstream of *amiE* led to measurable low constitutive amidase expression, and the realisation that the sequences upstream of the *amiR* gene somehow regulated *amiR* and that this then determined the amidase phenotype[33]. This was shown by construction of a hybrid plasmid with the *amiE* sequences from PAC433 and the *amiR* gene fragment from PAC1 — which showed constitutive amidase expression. It was thus concluded that a negative regulator gene was located upstream of *amiR* responsible for controlling either AmiR activity or expression. DNA sequencing and sequence analysis

identified *amiC*[33]. AmiC is a 385 amino acid polypeptide with codon usage similar to but less biased than that of *amiE* and *amiR*. In addition the *amiC* stop codon and *amiR* start codon were found to overlap (AUGA) thus possibly linking translation of the two genes within a polycistronic mRNA. It was thus concluded that amidase expression was positively regulated by AmiR via a transcription anti-termination mechanism and the activity of AmiR was negatively regulated by AmiC.

2.4.1. Studies of the Regulatory System

The whole of the *ami* insert from pAS20 was sub-cloned into pKT231 and mobilised into the *P. aeruginosa ami* deletion strain PAC452. This plasmid expressed high levels of inducible amidase activity — showing for the first time that it encoded all the information necessary for the provision of ligand-dependent inducible amidase activity. Additionally, the *amiC* gene was sub-cloned into broad and narrow host range vectors for complementation studies and the constitutive phenotypes of strains PAC111 and PAC142 were characterised as *amiC* mutations. The availability of cloned *amiC* and *amiR* genes also allowed the characterisation of strain PAC327 as an *amiC,amiR* double mutant. Use of broad host range, regulated expression (IPTG inducible) vectors carrying either *amiC* or *amiR* allowed the characterisation of previously isolated mutants but also gave some unexpected results. In the wild-type strain PAC1, low *amiR* expression *in trans* caused a low constitutive non-amide-induced, normal lactamide-inducible phenotype; and high *amiR* expression *in trans* caused a high constitutive phenotype. Thus overexpression of AmiR could titrate out the repressing effect of AmiC. In the *amiC* constitutive mutants high *amiC* expression *in trans* caused a marked reduction in amidase production that was not relieved by addition of the inducing ligand lactamide. AmiC was tested in vivo for interaction with the *amiE* promoter and was shown not to affect transcriptional regulation[36].

The accumulated evidence pointed to AmiC regulation of AmiR anti-termination activity by a direct protein–protein interaction either by covalent modification or sequestration. This was investigated in *E. coli* using a three-plasmid complementation system with each of the genes carried on a separate plasmid and the *amiC* and *amiR* genes expressed from vector promoters. In these investigations no regulated inducer-dependent amidase expression was seen. However, this was seen with plasmid pMW21, a broad host range vector carrying the sub-cloned *amiC,amiR* genes in their normal configuration downstream from a regulated vector promoter. In *E. coli* with a second plasmid carrying *amiE* and in *P. aeruginosa* PAC327 (*amiC,amiR*) the presence of pMW21 led to ligand-inducible amidase expression. It thus became apparent that for correctly regulated amidase expression the ratio of AmiC : AmiR had to be maintained and that this occurred with the wild-type gene configuration[36].

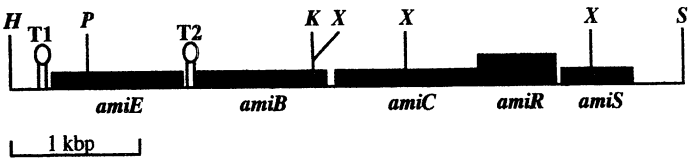

Figure 3. Gene organisation of the amidase operon of *P. aeruginosa*. Restriction sites for *Hin*dIII (*H*), *Pst*I (*P*), *Kpn*I (*K*), *Xho*I (*X*) and *Sal*GI (*S*) are shown as are the locations of the transcription terminators T1 and T2 and the genes.

2.5. AmiB and AmiS — Components of an Amide Transport System?

DNA sequencing of the region between *amiE* and *amiC* identified the *amiB* ORF; and that downstream of *amiR*, the *amiS* ORF[38]. AmiB is a 42 kDa polypeptide which contains an ATP binding motif and shares extensive homology with members of the Clp family of proteins. A maltose-binding-protein–AmiB fusion was shown to have an intrinsic ATPase activity, inhibited by ADP and ammonium vanadate. AmiS is an 18 kDa protein with a high content of hydrophobic residues. Hydropathy analysis indicated six transmembrane helices strongly suggesting that AmiS would be found within cells as an integral membrane protein. Taken together AmiB and AmiS would appear to be two of the components of an ABC transporter system. However, it is doubtful if aliphatic amide uptake requires a transport system and inactivating mutations in *amiB* or *amiS* have no effect upon amidase operon expression or cell growth on amides.

Thus the complete gene order of the amidase operon of *P. aeruginosa* is *amiE* — encoding the amidase enzyme; *amiB* — encoding an ATPase; *amiC* — encoding the operon negative regulator; *amiR* — encoding the operon positive regulator; and *amiS* — encoding an integral membrane protein (Figure 3).

2.6. Transcriptional Analysis of the Operon

To investigate the transcription anti-termination regulatory system, primer extension and S1 mapping were carried out. These studies identified the transcription start-point, confirming that the proposed promoter was functional and additionally showed high-level transcription in cells under all growth conditions — a requirement for an anti-termination regulatory system. Northern blotting showed the presence of a short transcript produced in cells grown under non-inducing conditions and both a 1.3 kb *amiE* transcript and a 5.0 kb complete operon transcript produced in cells grown under inducing conditions. In addition a second transcription terminator was identified

between the *amiE* and *amiB* genes which terminates approximately 50% of the *amiE* transcripts[34].

3. AmiC—STRUCTURAL STUDIES

AmiC was overexpressed from a regulated expression vector in *P. aeruginosa* PAC452 (*amiΔ*) and purified to homogeneity[33]. The purified protein was used for ligand binding studies with labelled acetamide and the K_D determined for inducers acetamide (3.7 μM), propionamide (3.1 μM) and lactamide (0.37 mM), and the amide analogue represser butyramide (0.37 mM)[36]. Homology searches of the SwissProt database with AmiC at this time showed a relatively weak match with BraC, the periplasmically located branched-chain amino acid binding protein from *P. aeruginosa* and several functionally related proteins. AmiC showed a general overall similarity to this family of proteins over its entire length with conformationally important residues being frequently conserved. Secondary Structure Predictions and Threading Analysis indicated that AmiC could adopt a three-dimensional structure similar to LivJ and other members of the small-molecule binding protein family. Recent homology analysis using AmiC has identified several protein sequences from a variety of bacteria including a putative two-component sensor kinase of *A. tumefasciens* [AC; NP396465]. *A. tumefasciens* also encodes an enantioselective amidase and the possibility exists that this AmiC homologue is involved in regulation of the amidase. Several nitrile hydratase regulatory proteins in various other organisms were also detected. In addition, the NCBI Conserved Domain Database identified the LivK [COG0683] domain in AmiC. The LivK family of

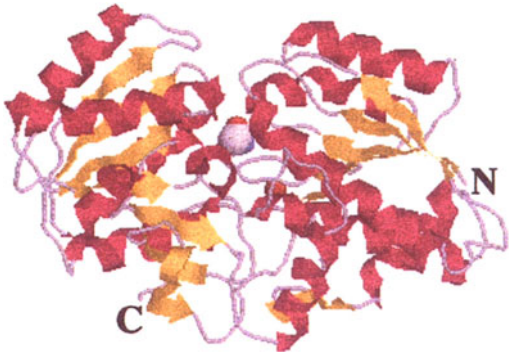

Figure 4. Secondary structure cartoon of AmiC-acetamide. The C-domain is on the left and the N-domain on the right. α-helices are coloured red, β-strands yellow, coils and loops grey. The bound acetamide lies between the domains and is shown as spacefill[27].

proteins are ABC-type branch-chain amino acid transporters — involved in the uptake of amino acids such as Leucine, Isoleucine and Valine.

Crystals of AmiC were obtained and the structure determined[27, 35]. Following the predictions AmiC was found to consist of two domains with an alternate β–α–β topology (Figure 4). The two domains are separated by a central cleft at the domain interface with an overall fold very similar to that of LivJ despite only 17% sequence identity. AmiC crystallised in a closed conformation with acetamide bound which significantly stabilised the structure — despite the ligand not knowingly being present in any of the buffers or the cell growth medium.

4. TRANSCRIPTION ANTI-TERMINATION IN VIVO

Studies replacing the *ami* promoter with that of Phage T7 or the *lac* operon showed that the anti-termination reaction was both promoter and RNA polymerase independent and apparently just an interaction between the leader RNA sequences and AmiR. This was investigated in *E. coli* with plasmids using a titration assay in which high AmiR-dependent amidase expression was reduced by provision *in trans* of excess leader transcript. The ability of the excess leader to titrate out the AmiR available for the anti-termination reaction and cause reduced amidase activity showed a direct interaction of the two components[37]. In vitro mutagenesis of the leader region was carried out to further investigate the RNA/AmiR interaction. The role of the leader ORF was investigated first by insertion of an internal stop codon, and second by changing the reading frame to nullify the normal stop codon. Neither of these changes affected the regulated expression of the *amiE* gene and we believe that the leader ORF has no biological role. The length of the leader RNA transcript is 100 residues to the run of Us following the Rho-independent terminator. It was envisaged that AmiR would interact with the RNA in such a way as to prevent formation of the termination stem/loop and would thus initially interact with RNA sequences upstream of the stem/loop. Targeted mutagenesis has identified two blocks of sequence centred at 27 residues and 8 residues before the start of the stem/loop required for AmiR interaction and anti-termination (Figure 5).

5. THE AmiR PROTEIN

The *amiC* gene had been sequenced, the protein overproduced and its structure determined in the presence of the inducing ligand acetamide. AmiR was much more difficult to investigate. Its biological role had been studied in vivo but all attempts to isolate the protein failed. The construction of an

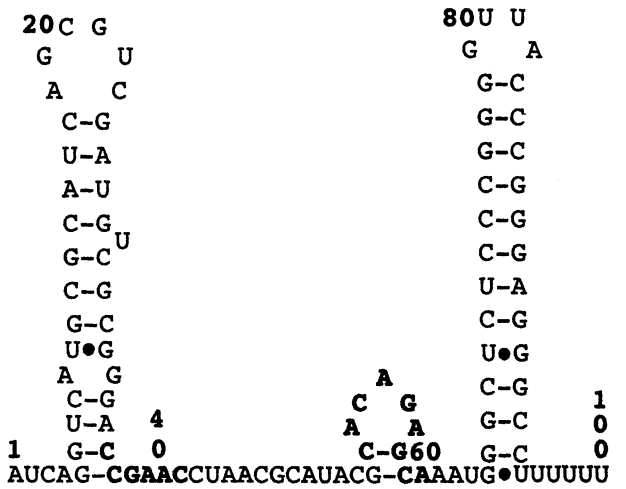

Figure 5. RNA-fold of the leader RNA transcript residues 1–100. Complementary residues are shown by lines and G–U interactions by filled circles. Residues shown to be involved in the AmiR/RNA interaction are shown in bold[18, 19, 37, 39].

MBP–AmiR fusion whilst not allowing the isolation of AmiR had been used for the production of a polyclonal antibody — used as a detection system. The only time that AmiR had been seen to be overproduced, or in fact at all using SDS PAGE, was using a T7 polymerase driven expression system and this resulted in large quantities of insoluble aggregated material found in the precipitate.

The available evidence pointed to AmiC as the ligand-sensor regulator that controlled the activity of AmiR — the anti-termination factor. It was apparent that a direct protein/protein interaction occurred between the two regulators and that for a normal inducible phenotype it was important that the wild-type stoichiometry was maintained. If the normal regulatory mechanism was by sequestration then it might be that an AmiC/AmiR complex formed under non-inducing conditions and the presence of inducing ligands would cause an AmiC structural change, release of AmiR, AmiR/RNA interaction and anti-termination.

This was investigated by Richard Norman using PCR to isolate the *amiC,amiR* genes from the wild type and a variety of interesting regulatory mutants.

6. THE AmiC/AmiR COMPLEX

The *amiC,amiR* genes were cloned from the wild-type PAC1; PAC111 — a high constitutive (but sensitive to butyramide repression) mutant; and

PAC153 — a formamide inducible mutant. The cloned genes were inserted into a broad host range, regulated expression vector and phenotypically characterised in PAC327 (*amiC,amiR*). Initial studies focused on the PAC111 genes. With the amidase regulatory system it is easy to imagine how constitutive mutants can arise. Changes to the DNA sequence of the *amiC* gene that disable the ORF but do not affect downstream transcription or translation of *amiR* will do the job. But a constitutive mutant still sensitive to butyramide repression, must mean that AmiC repressor function is lacking under non-inducing growth conditions, but the polypeptide must retain sufficient structural integrity to adopt its normal repressing configuration in the presence of the co-repressor butyramide. SDS-PAGE analysis of cell-free extracts of PAC452,pRAN111 showed the presence of a major AmiC band in the soluble fraction and a smaller AmiR band in the insoluble fraction after cell disruption and centrifugation. This is a repeat of earlier observations showing AmiC as soluble and easily isolated, and AmiR as insoluble and aggregating. The strain was then grown in the presence of butyramide and the products analysed. This time AmiC and AmiR were both found in the soluble fraction. Butyramide locked AmiC and AmiR together and prevented AmiR from forming insoluble aggregates.

6.1. The Wild-Type AmiC/AmiR Complex

What worked for the PAC111 regulatory complex should work for the wild type — and so the PAC1 AmiC/AmiR-butyramide complex was isolated and purified to homogeneity[22, 37].

The three questions that we hoped to answer with this complex were:

- What happened to the complex when inducing ligands were added?
- Could we get AmiR/RNA interaction in vitro?
- Could we determine the AmiC/AmiR and AmiR/RNA complex structures?

Gel filtration analysis showed that the AmiC/AmiR-butyramide complex had an M_R of 130 kDa and thus most likely comprised an AmiC dimer and an AmiR dimer. Extensive dialysis showed that the complex once formed was stable even if the butyramide was removed. Additions of increasing concentrations of acetamide to the butyramide complex analysed by gel-filtration chromatography showed complex dissociation into high M_R AmiR fractions and a mixture of AmiC dimers and trimers. Using a number of different acetamide concentrations and measurement of the percentage of complex dissociation, a K_D of 2.5 μM acetamide was obtained, close to the value obtained for acetamide binding to AmiC. Thus at the acetamide concentration at which 50% of the ligand binding sites of AmiC are filled the complex is 50%

dissociated and immediately suggests the way in which ligand mediated regulation of AmiR anti-termination activity occurs. The mechanism must explain what happens to the system under three types of cellular growth condition:

- Non-inducing growth conditions — small amounts of ligand-free AmiC/AmiR complex present.
- Inducing growth conditions — increased amounts of AmiC and AmiR produced with acetamide binding to AmiC causing complex dissociation and freeing AmiR for anti-termination.
- Repressing growth conditions — the presence of butyramide locking AmiC and AmiR together as an inactive complex.

With the purified AmiC/AmiR-butyramide complex we had examined stages three and two and also shown that stage one was a stable entity.

6.1.1. AmiC/AmiR Stoichiometry

The amidase system has evolved to be appropriately sensitive to amide growth substrates in the medium, but resistant to spurious induction caused by small amounts of endogenously produced amides. This is regulated by the AmiC:AmiR ratio which if unbalanced leads either to loss of inducibility or constitutive operon expression. During the isolation of the AmiC/AmiR complex the main contaminant was free AmiC present in excess. The normal AmiC:AmiR ratio was determined by scanning densitometry of cell extracts in which the plasmid located genes (in their normal configuration) were overexpressed. Standardisation was achieved by comparison with a washed, solubilised AmiC/AmiR crystal (see below) containing equimolar amounts of the two components. The AmiC:AmiR ratio in cells was calculated to be 3.3:1. Thus AmiC is always present in excess presumably to ensure — in the absence of acetamide — that the AmiR is complexed and inactive. This situation can be perturbed by provision of excess AmiR *in trans*. To investigate this further we established an in vivo titration system using PAC1,pSW40 in which addition of increased concentrations of IPTG leads to increased AmiR production in trans which then leads to increased amidase (and AmiC and AmiR) synthesis. The results with PACl,pSW40 showed a direct linear relationship between free AmiR — measured by western blotting, and operon expression — measured as amidase SA. Plasmid pSW40 is a broad host range vector carrying the *amiR* gene downstream from a regulated *tac* promoter. Addition of IPTG up to 0.1 mM causes increased *amiR* expression. The production of additional AmiR *in trans* would be expected to initially have little affect as small amounts of AmiR would be inactivated by complex formation with the molar excess of available AmiC. Production of more AmiR as a consequence of increased IPTG addition will eventually lead to saturation of the available AmiC and thus to

AmiR dependent operon expression — all in the absence of amide ligands. This will result in increased amidase synthesis and in turn the production of more AmiC and AmiR in the fixed 3.1 : 1 ratio. Synthesis of additional AmiC will lead to a decrease in the amount of AmiR available for anti-termination and cause reduced operon expression. However, the situation should soon stabilize at the new level of AmiR provided *in trans*. The results showed a linear relationship between the amount of free AmiR and the level of operon expression[22]. This would be predicted for a system regulated by a steric hindrance mechanism.

The availability of the AmiC/AmiR-butyramide complex, its dissociation by addition of actemide and the synthesis in vitro of labelled *ami* leader RNA run-off transcripts allowed us to study the AmiR/RNA interaction. An AmiR sequence-specific RNA binding activity was detected with a K_D of 1.0 nM. This was shown by RNA bandshift studies using varying AmiR or leader transcript concentrations and the use of cold-specific competitor RNA.

6.1.2. amiC,amiR Translational Coupling

The AmiC:AmiR ratio determined in vivo is thought to be maintained by a translational coupling mechanism. The amidase regulator genes are linked such that the stop codon of *amiC* and the start codon of *amiR* overlap (**AUGA**) and they lie within a polycistronic transcript during all growth conditions. Under non-inducing growth conditions residual read-through of the T1 terminator ensures that low levels of amidase are always present to hydrolyse endogenously produced amides and prevent spurious induction. This low level transcription will be 50% reduced by the T2 terminator between *amiE* and *amiB* but will continue into *amiB, amiC, amiR* and *amiS*. This must provide sufficient AmiC and AmiR for induction in the presence of added amides. Under inducing growth conditions there will be large-scale production of AmiC and AmiR with the AmiR mainly complexed to the leader RNA. We have proposed that a translational coupling system operates such that *amiR* is only translated by re-initiation after translation of *amiC* and the stoichiometry is thus determined by the re-initiation frequency. The S/D sequence of *amiC* shows a much higher homology to the 3′ end of the 16S rRNA than that of the *amiR* S/D sequence and RNA secondary structure predictions of the respective translation initiation regions show potential secondary structure formation (Figure 6). However, the *amiC* leader secondary structure is relatively unstable (−4.4 kcal/mol) and the S/D sequence is ribosome accessible, whereas the potential *amiR* leader secondary structure is much more stable (−13.8 kcal/mol) and the S/D sequence sequestered into a secondary structure stem/loop formation. It is thus an attractive hypothesis that *amiR* is only translated by re-initiation after translation of *amiC* and the cell uses this mechanism to maintain the AmiC:AmiR ratio.

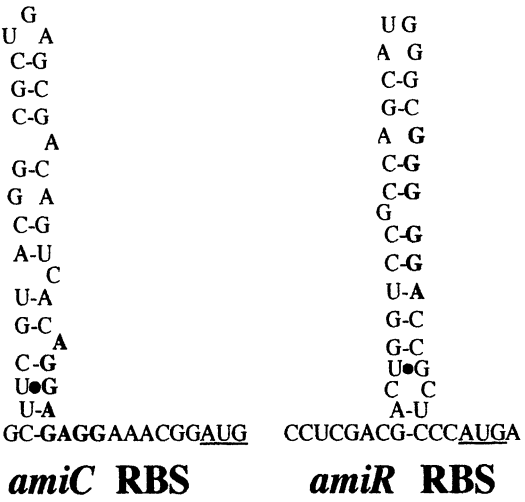

Figure 6. RNA folds of the *amiC* and *amiR* gene translation initiation regions. Complementary residues are shown by lines, G–U interactions by filled circles and the S/D sequences are shown in bold[18, 19, 22, 39].

6.2. AmiC/AmiR-Butyramide Complex

The AmiC/AmiR–butyramide complex crystallises with an AmiR dimer and two AmiC monomers in the asymmetric unit[24]. The size of the complex isolated as measured by gel-filtration chromatography of ~130 kDa indicates that the asymmetric unit represents the in vivo solution state.

6.2.1. AmiR: Structure and Function

AmiR is present in the complex as an intimate dimer of the 196 residue monomer. The AmiR N-terminal domain consists of a doubly wound α–β–α sandwich constructed around five parallel β-strands with a *b-a-c-d-e* topology. Extending from this domain is a long α-helix C-terminal domain of 55 residues length. The first 30 residues participate in a coiled-coil interaction with the equivalent helix from the other dimer subunit and the C-terminus of this helix participates in a three-helix bundle involving the remainder of the chain (Figure 7).

The N-terminal domain of AmiR is structurally homologous to the response regulator "receiver" domain of bacterial two-component signal transduction systems[26]. However, it lacks the conserved phosphate acceptor residues normally associated with this group of structurally related proteins. Within this functional group the C-terminal domains represent the "output" domain and structural types and functions vary across the several hundred members studied. It thus seemed most likely that the anti-termination activity of AmiR would reside in the C-terminal domain and deletion of the last helix

Figure 7. Cartoon structure of AmiR. N-terminal globular domain—response regulator "receiver" domain (dark blue-blue-dark green), central coiled domain (light green-yellow-orange) and C-terminal three-helix bundle (dark orange-red)[24].

and last two helical regions cause a loss of anti-termination activity in vivo. This supports the argument that AmiR is functionally the response regulator and that RNA binding resides in the C-terminal domain.

Homology searches and structural predictions have identified the AmiR C-terminal domain as the prototype for a >40 member RNA-binding ANTAR family. Family members are spread through the Bacterial Kingdom and include important pathogens and symbionts. In each case it is presumed that the identified ORF has the ANTAR motif as an RNA-binding response regulator output domain and that regulation is mediated by a type of anti-termination mechanism[29]. Both the AmiR domain (CD; COG3707.1AmiR) and the ANTAR domain (CD; pfam03861.4 ANTAR) are identified in the Conserved Domain Database. Recent database searches using AmiR have found sequence homology with several putative response regulators, anti-terminator proteins and uncharacterised hypothetical proteins.

6.2.2. AmiC/AmiR–Butyramide Complex Structures

The AmiC/AmiR–butyramide complex contains an AmiR dimer and two molecules of AmiC[24]. The calculated M_R is close to that of the complex isolated from cells and measured by gel-filtration and thus represents the biologically active state. The two AmiC molecules found in the complex bind to the "top"

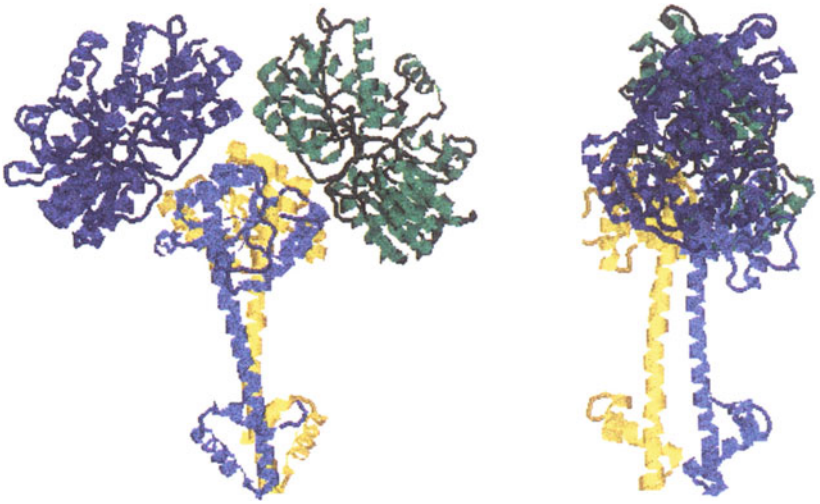

Figure 8. Cartoon of the AmiC/AmiR-butyramide structure. Arrangement of the AmiC (blue/green/blue) and AmiR (pale blue/yellow) molecules in the AmiC/AmiR complex[24].

surface of the AmiR dimer interacting simultaneously with both molecules equally (Figure 8). There is little AmiC/AmiC interaction and AmiC binding to the AmiR dimer can be regarded as independent. Residues from both AmiC domains of each AmiC molecule make contact with both subunits of the AmiR dimer. The ligand-free version of this complex isolated after extensive dialysis is predicted to have an identical structure.

6.2.3. AmiC-Butyramide Structure

Butyramide binds to AmiC making similar contacts to that of acetamide in the ligand-binding pocket. The major difference between the AmiC-butyramide structure seen here and that of the AmiC-acetamide structure determined previously is a slight inter-domain movement between the AmiC N- and C-terminal domains to accommodate the larger butyramide molecule. In addition there are a number of water molecules within the inter-domain cleft (see below).

6.3. Molecular Mechanism of the Induction Process

Addition of acetamide to the AmiC/AmiR-butyramide complex showed displacement of the butyramide by acetamide and complex dissociation. This is representative of the in vivo induction process and a comparison of the AmiC-acetamide structure determined previously, with the AmiC-butyramide structure from the complex allowed us to study the transition. The major

difference between the closed structure of AmiC-acetamide and the open structure of AmiC-butyramide in the complex is the relative orientation of the N- and C-terminal domains. In the closed conformation the opposing faces of the domains of AmiC are in direct contact. The walls of the domains are completely closed down around the ligand, and the size of the pocket thus determines the inducer specificity of the system. In the presence of butyramide or absence of acetamide there is a small inter-domain movement such that a layer of water molecules forms between the side-walls and the size of the ligand-binding pocket increases — enough to accommodate butyramide[24]. The affinity of AmiC for butyramide is 100 fold less than that for acetamide but the presence of the co-repressing ligand allowed us to isolate the complex since butyramide locks AmiC into the "open" conformation. AmiC and AmiR can only form the complex with AmiC in the open conformation. The AmiC surface residues from either side of the inter-domain cleft make a set of highly complementary interactions with the "top" surface of the AmiR dimer. This interaction — and complex formation — is absolutely dependent upon the AmiC open conformation. Thus addition of acetamide will lead to displacement of butyramide from the ligand-binding site, closure of the AmiC domains around the acetamide and complex dissociation — since closure of the cleft causes breakage of the AmiC–AmiR interactions which stabilize the complex.

7. STUDIES OF AMIDASE REGULATORY MUTANTS

The DNA sequences of the *amiC,amiR* genes from strains PAC111, PAC153 and PAC181 have been determined and compared to the wild type. These studies showed that the previously published *amiR* sequence was incorrect and that residue 64 was R and not G. This residue was visible in the AmiC/AmiR complex structure.

PAC111, a high constitutive mutant sensitive to butyramide repression, carries a 6 bp deletion removing AmiC residues D317 and I318. These are located on a surface loop on the N-terminal domain on the opposite side and far from both the amide binding cleft and the AmiR dimer. The wild type in this region shows a three-turn α-helix formed by residues 308–315, linked by a 5-residue loop to an anti-parallel β-strand. Structural predictions show that the two-residue deletion would cause a shortening of the helix by half a turn but this would not be expected to affect the hydrophobic core of AmiC since the overall positions of the helix and β-strand are the same (R.A. Norman and R.E. Drew, unpublished data). However, it is apparent from the strain phenotype that the two-residue deletion must change the overall AmiC structure in

such a way that in the absence of inducing ligands the normal AmiC/AmiR interaction is disrupted so that the AmiR/RNA anti-termination reaction occurs — seen as constitutive operon expression. In addition the structure of the C11 AmiC molecule must retain enough of the wild-type characteristics such that the AmiC/AmiR complex forms in the presence of butyramide.

PAC153 is a formamide (and acetamide) inducible mutant and carries two mutations an AmiC R197C substitution and an AmiR P91S substitution. The AmiC mutation occurs in a surface loop in the C-terminal domain far from the amide-binding site, and away from the sites of the AmiC/AmiR interactions. The AmiR mutation occurs in a helix-cap residue whose Cγ and Cδ side-chain atoms pack against the face of the side-chain ring of AmiC Fl11 as part of the major cluster of interactions between AmiC and AmiR in the complex. The P→S substitution would result in the loss of favourable hydrophobic interactions and lead to a decrease in the stability of the AmiC/AmiR complex relative to wild type. Thus the smaller interaction energy afforded by the binding of formamide rather than acetamide to AmiC must then be able to outweigh the decreased stability of this complex and cause operon induction[24].

PAC181 is a butyramide inducible mutant that produces the B enzyme and carries two mutations, one the *amiE* S7F substitution which leads to production of an amidase enzyme with a higher rate of butyramide hydrolysis and a second regulatory mutation leading to butyramide inducibility[25, 32]. This strain was isolated after a number of mutagenic treatments, a genetic cross and a further selection. DNA sequencing identified an *amiC* mutation (T106N) as responsible for butyramide inducibility. The AmiC protein was overproduced and isolated from PAC452,pSW1181 in the presence of butyramide. The crystal structure of the PAC181 AmiC-butyramide was determined[25]. The structure showed electron density for asparagine at residue 106 confirming the DNA sequence data, and electron density for a bound butyramide whose amide head-group and α-carbon are in an identical position to the equivalent atoms in acetamide bound to the wild-type AmiC. The Thr→Asn mutation removes part of the side-wall of the amide binding pocket and increases the volume of the ligand-binding site. Butyramide is bound here in an unfavourable folded-over conformation with the γ-methyl group in van der Waal's contact with the plane of the amide head group. In addition, the amide oxygen of Asn106 is buried in a hydrophobic environment with no hydrogen bonds to the rest of the protein or any solvent molecules. This would be expected to be highly unfavourable and the melting temperature measured in the near-UV CD for denaturation of the protein is down-shifted 20 K compared with the wild type AmiC-butyramide. Thus the selective pressure to which this mutant was the response required only that the system became butyramide-inducible at a sufficient level to fulfill its biological role and its absolute structural stability needed not be optimal.

In addition to these investigations a study of the ligand-binding site of the wild-type AmiC protein showed that of the mixed (R and S) isomers of lactamide used as gratuitous inducer of the system, only S-lactamide could fit into the ligand-binding site of AmiC. The availability of the separated isomeric forms, and their use in induction studies in vivo confirmed this observation (G. Siligardi and R.E. Drew, unpublished data).

8. AmiR AND TRANSCRIPTION ANTI-TERMINATION

The investigations of the amidase operon have identified AmiR as the response regulator, a site-specific RNA-binding protein functional as a transcription anti-termination factor. Its activity is regulated by a sequestration mechanism by complex formation with the negative regulator AmiC. The RNA-binding motif has been identified from analysis of deletion mutations as the C-terminal three-helix bundle — a region that is fully solvent exposed in the AmiC/AmiR complex. It is thus apparent that upon release from the complex that AmiR must subsequently either oligomerise or undergo a structural isomerisation to gain functionality. This has been investigated using site-directed mutagenesis. The problem with the analysis of the results is the determination of whether the changes made affect the polymerisation reaction and thus the subsequent RNA binding or whether they just affect the RNA binding the only facility that we can measure.

It is not clear at this time whether AmiR binds transiently to the leader RNA sequences to prevent termination loop formation and is then recycled or whether it remains permanently associated with the transcript. In the latter case there would be a requirement for higher level AmiR production — for an AmiR multimer binding to each mRNA transcript. Although not investigated so far it also remains a possibility that the catabolite repression affect shown in the early physiological studies is mediated through the AmiR/RNA interaction causing increased/decreased mRNA stability rather than by a direct promoter/RNA polymerase affect.

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16

TRANSCRIPTIONAL REGULATION AND CATABOLIC STRATEGIES OF PHENOL DEGRADATIVE PATHWAYS

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INTRODUCTION

Microorganisms that catabolise phenol and methylated phenols serve as excellent model systems for addressing molecular mechanisms of adaptation and the limitations imposed by both regulatory and biochemical properties of bacteria[69]. The capacity of microbial systems to catabolise organic matter has co-evolved with the presence and availability of the given compounds in the environment. Phenol and methylated phenols are relatively simple aromatic compounds that are naturally produced through microbial activities and the breakdown of plant material. Thus, many different microorganisms that are capable of efficient and complete catabolism of these compounds are readily isolated. However, as with catabolism of other aromatic compounds, the efficiency with which different phenolics can be degraded is influenced by many factors, including the prevailing physicochemical conditions, the concentration/toxicity load, and the nature and the location of substitutions on the aromatic ring. The relatively recent supplement of naturally occurring phenolics by large quantities of man-made phenol and derivatives that harbour substituents that are not encountered in nature has in some cases resulted in inefficient removal from the environment. Consequently, phenols feature high on the watchdog list of environmental pollutants (www.environmentaldefense.org), and the regulatory

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devices and biochemical activities of bacteria involved in their catabolism have potential utility in counteractive bioremediation protocols[69].

In addition to the specificities and catabolic capacity of the pathway enzymes, regulatory mechanisms can influence aromatic catabolism, leading to sub-optimal performance in some instances. First, aromatic compounds in most cases directly modulate the activity of transcriptional regulators to control their own catabolism. Thus, regulator recognition of effector aromatics plays a key role in transcriptional output. In addition, aromatic–catabolic systems have to function efficiently within the context of the host and be globally integrated so as to avoid detrimental energy-fluxes that would otherwise compromise fitness and survival. Adaptation to nutritional and other changes in the environment requires the assimilation of multiple signals for a coordinated regulatory response. Transcriptional regulatory properties of pathway gene expression are key components through which multiple environmental signals can be integrated. Hence, both specific regulatory circuits and dominant global regulation in response to host physiology have major impact on expression levels of pathway enzymes[68]. This chapter aims to highlight recent progress and limitations in our understanding of (a) the relationship between the enzymatic capacity of pathways and the cognate transcriptional regulation strategies, and (b) the interplay between the environment and the host bacteria, that jointly impact on the expression and thus performance of aerobic phenol catabolic systems. While the primary focus of the chapter is on phenol catabolic pathways, in particular that of *Pseudomonas* sp. CF600 that represents a predominant genotype in the environment, many of the enzymatic and regulatory mechanism described are also pertinent for other systems.

2. GENETIC ORGANISATION AND BIOCHEMICAL ROUTES FOR PHENOL CATABOLISM

Pseudomonads and related Gram-negative bacteria are able to utilise phenolics as sole carbon and energy sources by virtue of pathways encoded by one or more operons that can be located on either plasmids or the chromosome. The clustering of genes into functional operons is the evolutionary consequence of the bacterial strategy of channelling diverse aromatic compounds into one of a limited number of central pathways[89]. Within the general bacterial strategy for aerobic catabolism of aromatic compounds, distinct enzyme systems insert one or more oxygen atoms to convert initial pathway substrates to one of a few destabilised key intermediates, usually catechol or substituted catechols. The two hydroxyl groups of these key intermediates destabilise the aromatic ring, making it amenable to cleavage by ring fission dioxygenases[21].

Table 1. Summary of the function of *dmp*-encoded proteins.

Gene	Product		Function
	Size (aa)	Name	
Multicomponent phenol hydroxylase/monooxygenase			
dmpK	92	P0 or DmpK	Auxiliary assembly-assisting protein[46, 59]
dmpL	331	P1 or DmpL	Oxygenase subunit[15, 46, 61]
dmpM	90	P2 or DmpM	Stimulatory subunit[14, 46, 61]
dmpN	517	P3 or DmpN	Oxygenase subunit[15, 46, 61]
dmpO	119	P4 or DmpO	Oxygenase subunit[15, 46, 61]
dmpP	353	P5 or DmpP	Reductase subunit[46, 61]
Meta-cleavage pathway enzymes			
dmpQ	112	DmpQ	C23O reactivating ferredoxin-like protein[31, 60]
dmpB	307	C23O	Catechol 2,3-dioxygenase[10]
dmpC	486	HMSD	2-Hydroxymuconic semialdehyde dehydrogenase[47]
dmpD	283	HMSH	2-Hydroxymuconic semialdehyde hydrolase[47]
dmpE	261	OEH	2-Oxopent-4-dienoate hydratase[73]
dmpF	312	ADA	Aldehyde dehydrogenase (acylating)[58, 73]
dmpG	345	HOA	4-Hydroxy-2-oxovalerate aldolase[58, 73]
dmpH	264	4OD	4-Oxalocrotonate decarboxylase[73]
dmpI	63	4OI	4-Oxalocrotonate isomerase[73]

Catechol ring fission dioxygenases cleave the aromatic ring either adjacent to the hydroxyl groups (extradiol *meta*-cleavage) or between the two hydroxyl groups (intradiol *ortho*-cleavage). The non-aromatic ring cleavage products are then sequentially transformed to substrates of mainstream metabolism, namely pyruvate and acetyl-coenzyme A (CoA) by the *meta*-cleavage pathway (Table 1, Figure 1), and succinyl- and acetyl-CoA by the *ortho*-cleavage pathway.

2.1. Initial Hydroxylation of Phenol

Pseudomonads and microorganisms from other Gram-negative and -positive bacterial genera and yeast can all utilise flavoprotein phenol hydroxylases to accomplish the comparatively simple task of destabilising oxygen-rich phenol (Figure 2). In these single- or two-component flavoproteins, the flavin moiety is used to activate molecular oxygen that is otherwise unreactive with organic compounds (see Vol. 3 for detailed reviews on reaction mechanisms of oxygenases of aromatic compounds). Thus it was unexpected when *Pseudomonas* sp. CF600 was first found to utilise a large multicomponent phenol hydroxylase that had low but significant homology to enzymes involved in monooxygenation of unactivated compounds such as methane and

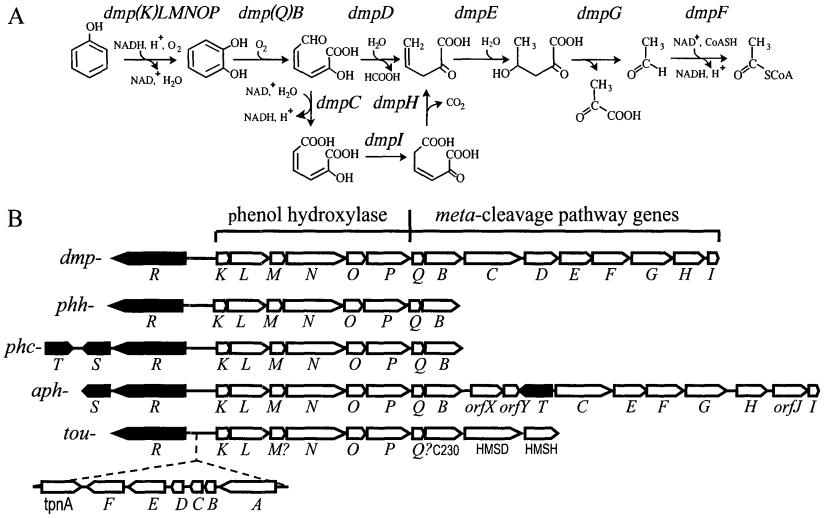

Figure 1. A Biochemical route for phenol catabolism, encoded by the *dmp*-system of the pVI150 catabolic plasmid of *Pseudomonas* sp. CF600 that involves a multicomponent phenol hydroxylase and a subsequent *meta*-cleavage pathway. Both the *meta*-pathway hydrolytic (*dmpD*-encoded) branch for dissimilation of compounds catabolised through 3-methyl and 3,4-dimethylcatechol, and the 4-oxalocrotonate (*dmpCIH*-encoded) branch for dissimilation of compounds through catechol and 4-methylcatechol, are shown. See Table 1 for enzyme designations. B Schematic illustration of the genetic organisation of the *dmp*- (*Pseudomonas* sp. CF600: X68033, M60276, X60657, M33263, X52805, X60835), *phh*- (*Pseudomonas putida* 35X: X79599, X79063), *phc*- (*Comamonas testosteroni* R5: AB061422, AB050891, AB024741), *aph*- (*C. testosteroni* TA441: AB008787, AB006480, AB006479, AB029044), and *tou*- (*Pseudomonas stutzeri* OX1: AJ005663, AJ309239) systems. For the *tou*-system, DNA sequence information is available for *touR*, the *touABCDEF* cluster, *touK*, and part of *touL*; the location and function of the remainder of the genes indicated is inferred from gene hybridisation and/or enzyme assays[5]. The *touABCDEF* cluster, which is spanned by remnants of an apparent transposition event(s), is shown as an insertion into a pre-existing *dmp*-like system[9]. Regulatory genes are shown in black, see Table 2 for family designations of the cognate regulators.

toluene that lacks an electron donating hydroxyl (reviewed in [60]). In these large multicomponent systems, an auxiliary reductase is required to transfer electrons to the oxygenase active site (Figure 2). In the case of *Pseudomonas* sp. CF600, this multicomponent phenol hydroxylase permits growth at the expense of phenol, mono-methylated phenols (cresols), and 3,4-dimethylphenol.

All the six phenol hydroxylase associated genes of *Pseudomonas* sp. CF600 (*dmpKLMNOP*, Table 1, Figure 1B) are required to form the active enzyme in vivo. However, only the DmpLMNOP polypeptides are contained within the active enzyme complex, and individual gene deletions demonstrated that each is essential for activity[46, 61]. The role of DmpK lies not within the

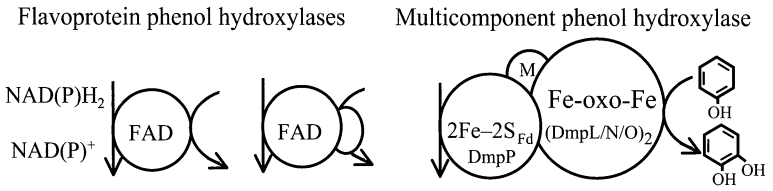

Figure 2. Schematic illustration of phenol hydroxylating enzymes of bacteria. Single component flavoproteins are exemplified by the TbuD phenol hydroxylase of *Ralstonia pickettii* PKO1[36] and the pEST1126 encoded *PheA*-system of *Pseudomonas* EST1001[48]. Examples of two component flavoprotein systems are the flavoprotein component PheA1 and the small but essential PheA2 protein of *Bacillus thermoglucosidasius* A7[24] and similar proteins of the chlorophenol hydroxylase of *R. pickettii* DTP0602 (accession number D86544). Multicomponent phenol hydroxylases are exemplified by the pVI150 encoded *dmp*-system of *Pseudomonas* sp. CF600[15]. Other examples of phenol degradative pathways that utilise Dmp-like multicomponent phenol hydroxylase are given in the text and Table 2.

enzyme activity *per se*, but rather in assembly of the active form of the oxygenase within the cell, and DmpK can catalyse reactivation of inactive enzyme in vitro in the presence of Fe[59]. Upon purification, the five polypeptides of the phenol hydroxylase are resolved into three components: DmpP, DmpL/N/O, and DmpM. The DmpP reductase contains a flavin adenine dinucleotide and a ferredoxin-like [2Fe–2S] centre. The flavin of DmpP is presumed to accept electrons from the obligate two-electron donor NAD(P)H and pass them one at a time to the active site of the oxygenase via the [2Fe–2S] centre[61]. In its active form, the dimer of the three component oxygenase, $(DmpL/N/O)_2$, contains an oxo-bridged diiron centre liganded within DmpN[15]. However, the activity of the oxygenase is stimulated by the monomeric DmpM protein that interacts directly with the DmpN subunit and greatly enhances steady state turnover rates and product yield[14, 15, 62]. Thus the phenol hydroxylase from *Pseudomonas* sp. CF600 is functionally similar to other biiron centre monooxygenases such as the methane monooxygenases from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b[35], and toluene-2- and toluene-4-monooxygenases from *Burkholderia cepacia* G4 and *Pseudomonas mendocina* KR1[39, 56].

2.1.1. Prevalence and Substrate Specificity of Multicomponent Phenol Hydroxylases

Despite the fact that flavoproteins can mediate efficient growth at the expense of phenol, multicomponent monooxygenases encoded by homologues of the *dmp(K)LMNOP* genes of *Pseudomonas* sp. CF600 appear to be the most prevalent type of phenol hydroxylase found in environmental isolates (Figure 1, Table 2). Gene-probing analysis identified *dmp*-like phenol hydroxylases

genes in conjunction with a similar cognate regulator and *meta*-cleavage pathway genes in the archetypal phenol degrader *P. putida* U, and as the predominant genotype in phenol-degrading marine isolates[45]. Similarly, *dmp*-like phenol hydroxylases genes were also identified as the dominant genotype among bacteria isolated downstream from a major phenol contamination site, despite previous augmentation of the natural population with *Pseudomonas* harbouring the plasmid encoded *pheA* gene for a flavoprotein phenol hydroxylase[55]. In addition, DNA sequence analysis and PCR screening of numerous phenol degrading bacteria from various sources have identified genes homologous to *dmpKLMNOP* (Figure 1B). Thus, this complement of genes has been found in organisms isolated from a wide range of environments (e.g., sea- and fresh-water, fresh-water mud, aquifer soil, and activated sludge), and from diverse geographical locations (e.g., Estonia, Germany, Japan, Norway, UK, and USA). Hence, some selective advantage appears to have conserved the employment of the multicomponent phenol hydroxylase systems during global dispersal.

The requirement for each of the *dmpKLMNOP* genes for the active form of the protein provides a clear evolutionary force for inheritance or recruitment of the complete set of genes as a functional unit. To date, *dmpKLMNOP*-like genes have been found predominantly in association with *meta*-pathway genes, yet, since catechol can be further metabolised by either a *meta*- or *ortho*-cleavage pathways, it could be anticipated that complements of *dmp*-like phenol hydroxylase genes could be found in association with *ortho*-cleavage pathways. This is indeed illustrated by the *mop*-system of *Acinetobacter calcoaceticus* NCIB8250 (Table 2). However, as a consequence of the enzymology of the *ortho*-cleavage pathway, this combination would be limited and unable to further metabolise methyl-substituted catechols produced by the action of multicomponent phenol hydroxylases on methyl-substituted phenols.

The activities of bacterial phenol hydroxylases are frequently the rate limiting step in the catabolism of phenol[69]. The apparent kinetic parameters for phenol hydroxylation by the now extensive class of multicomponent phenol hydroxylases vary immensely[26, 86], and augmentation with genes encoding these multicomponent enzyme systems has been employed to significantly enhance turnover rates. For example, insertion of genes encoding a multicomponent phenol hydroxylase of high catabolic capacity from *C. testosteroni* R5 into a phenol degrading isolate improved the performance of phenol remediation by activated sludge[87]. In general, the specificities of multicomponent phenol hydroxylases are broad, and can differentially accommodate mono- and di-substituted methylphenols, chloro- and ethyl-substituted phenolics[43, 80]. Like some related toluene monooxygenases, a subgroup of the multicomponent phenol hydroxylases can also mediate efficiently co-metabolism of the toxic non-aromatic pollutant trichloroethylene[26, 27, 40]. The general broad

specificity of multicomponent phenol hydroxylases for different substituted phenols, and the ability of some of these enzymes to mediate co-metabolism of common potential co-pollutants, probably underlies the prevalence of *dmpKLMNOP*-like genes in phenol degrading isolates.

2.2. The *Meta*-Cleavage Pathway Enzymes

The classical work during the late 1960s and 1970s with the phenol degrader *P. putida* U was responsible for much of the early progress in elucidating the sequential steps and the roles of the two branches of the *meta*-cleavage pathway depicted in Figure 1A. The conclusions were subsequently validated and expanded on by biochemical and genetic studies of the *xyl*- and *dmp*-encoded *meta*-cleavage pathways (e.g., the *dmpQBCDEFGHI* genes; Table 1, Figure 1B). For example, DNA sequence analysis of the *dmp*-operon revealed one more gene than known biochemical steps. This finding prompted the hunt for a missing step in the *meta*-pathway that led to the discovery of the *dmpF*-encoded aldehyde dehydrogenase (acylating) activity[58, 73]. This enzyme mediates the last enzymatic step of the pathway (see Figure 1A) and provides an operon-controlled and energetically favourable mechanism for metabolism of acetaldehyde, which has subsequently been shown to have a counterpart in all *meta*-cleavage pathways in which the complete DNA sequence is known. All of the proteins of the *dmp*-pathway have been purified and their biological functions assigned on the basis of in vitro enzyme assays and growth profiles of pathway mutants defective in individual steps (Table 1)[69]. Both the *xyl*- and *dmp*-pathways involve the complete complement of *meta*-pathway genes that have isofunctional counterparts in other *meta*-cleavage pathways involved in catabolism of a whole variety of aromatic compounds[60]. The evolutionary and mechanistic implications of sequence homologies of *meta*-pathway enzymes genes have been described in detail previously[60, 89]. Therefore, here I only describe the enzymatic activities in relation to their roles in catabolism that probably underlie (a) the different complements of *meta*-pathway genes found, and (b) transcriptional regulatory strategies of the cognate pathways, that are described in the following sections.

The critical step of ring opening is catalysed by catechol 2,3-dioxygenase, an enzyme that is particularly sensitive to inactivation upon turnover on 4-(*para*)-substituted catechol. A small auxiliary ferredoxin-like protein is frequently encoded adjacent to the catechol-2,3-dioxygenase genes of *meta*-pathway operons (e.g., *dmpQ* in Figure 1B) and is intimately involved in regeneration of catechol-2,3-dioxygenase activity. These small auxiliary proteins catalytically reactivate through reduction of the iron atom in the active site of catechol-2,3-dioxygenase that inadvertently becomes oxidised[30, 31]. Loss of DmpQ[60], or its XylT homologue of the *meta*-pathway encoded by the TOL

plasmid pWW0[57], results in strains that are unable to grow at the expense of compounds that are catabolised through *para*-substituted methylcatechols (i.e., 4-methyl and 3,4-dimethylcatechol). Conversely, these strains can still grow on compounds that are catabolised through catechol and 3-methylcatechol. Thus, these auxiliary proteins are critical for the broad substrate range of *meta*-cleavage pathways[57].

Not all *meta*-cleavage pathways gene clusters possess the full complement of genes for both branches of the pathway. For example the *aph*-encoded system of *C. testosteroni* TA441 lacks the *dmpD* homologue for the hydrolytic branch (Figure 1B)[3]. This hydrolase-encoding gene provides metabolic versatility since it is required during growth on compounds that are catabolised through 3-methylcatechol. Conversely, the *tod*- and *bph*-encoded systems of *P. putida* F1 and *Pseudomonas* sp. KKS102, respectively, lack the *dmpCIH* homologues for the 4-oxalocrotonate branch that is utilised for catabolism of compound through 4-methylcatechol. The necessity for continual reactivation of catechol 2,3-dioxygenase during turnover on 4-methylcatechol, which is further catabolised by this branch, would provide a selective force for co-conservation of the *dmpQ* and *dmpCIH* homologues. Consistent with this idea, the *tod*- and *bph*-encoded systems that do not catabolise any compound through 4-methylcatechol lack both the *dmpCIH* homologues and the *dmpQ* homologue (reviewed in ref. [60]).

One of the most interesting aspects of the biochemistry of the *meta*-cleavage pathway is the tight physical association and interdependence of some of the pathway enzymes. Two enzyme pairs that each catalyse sequential steps in the pathway co-purify, namely the decarboxylase/hydratase (DmpEH) and aldolase/CoA-dependent dehydrogenase (DmpGF) pairs (see Figure 1, ref. [73] and references therein). In each case, the activity of the enzyme that makes the substrate for its partner is dependent on its partner for activity. In the aldolase/CoA-dependent dehydrogenase (DmpGF) example, cross-regulation at the level of enzyme activity occurs since the activity of the aldolase is dependent on the presence of the NAD^+ co-factor required by its partner dehydrogenase[58]. The recent crystal structure of the DmpFG complex has revealed a gated channel between the active sites of the two enzymes. Substrate conversion by DmpG and NAD^+ binding to DmpF has been proposed to provide conformational control points for the gates to sequester and mediate a unidirectional flow of the metabolite[38]. This level of interdependence suggests that metabolic channelling may be important for pathway efficiency. While traditional protein purification strategies readily identify tight physical interactions, it is possible that additional unidentified weaker physical associations may also contribute to efficient and controlled flow of pathway intermediates through the sequential steps. The selective advantages of the close linkage of the *dmpQB*/*dmpCIH* genes, the *dmpEH* genes, the *dmpGF* genes, and the added metabolic versatility provided by the *dmpD*-encoded

hydrolytic branch, accounts for the presence of all *meta*-pathway genes. In addition to an increased likelihood of inheritance of a functional metabolic module, it appears possible that efficient channelling of intermediary metabolites also contributes to the selective pressure that drives clustering of *meta*-pathway genes into operons.

3. TRANSCRIPTIONAL CONTROL OF PHENOL CATABOLISM

Transcriptional control provides the entry point that allows aromatic catabolic pathways to be specifically expressed in response to the presence of substrates and at levels that will provide a sufficient metabolic return. Aromatic compounds control the activity of specific members of many families of transcriptional regulators to direct expression of the specialised enzymes for their own catabolism. In all these systems the effector molecules, which can be either an initial aromatic pathway substrate or a non-aromatic pathway intermediate generated through low basal level transcription, provide the specificity of induction of gene expression[68]. The regulators of aromatic catabolism vary greatly in the range of compounds they respond to. For example, LysR-family members are generally restricted in their response, being activated by an intermediate of the corresponding catabolic pathway, for example, *cis, cis*-muconate for CatR[65], and 2-hydroxymuconic semialdehyde for AphT[3] (see Table 2). Regulators that respond to initial pathway substrates on the other hand, frequently have broader inducer specificity and can additionally respond to non-metabolisable structural analogues of the pathway they control. This is the case for the aromatic-responsive σ^{54}-dependent family of regulators that lie at the top of the hierarchy of transcriptional control of most aerobic phenol degradative systems, which also includes a member that potentially controls anaerobic phenol catabolism (Table 2).

3.1. Response Profiles of Aromatic-Responsive σ^{54}-Dependent Regulators

The σ^{54}-dependent regulator DmpR controls catabolism of phenol, mono-methylated phenols and 3,4-dimethylphenol through the Po promoter of the *dmp*-operon, while the closely related toluene/xylene responsive XylR controls the Pu promoter of TOL plasmid pWW0 (see Chapter 18). Both these aromatic-responsive regulators exhibit broad effector-response profiles. As illustrated in Figure 3, DmpR requires the hydroxyl group of phenol but in addition to methylphenols, this regulator can also respond to some structural

Table 2. Representative transcriptional regulators of phenol catabolic pathways.

Regulator/pathway[a]	Host (plasmid)	Accession[b]
σ^{54}-dependent family (activators)		
AphR/Phenol (LmPH, *m*)	*C. testosteroni* TA441	BAA34177
DmpR/Methylphenols (LmPH, *m*)	*Pseudomonas* sp. CF600 (pVI50)	CAA48174
MopR/Phenol (LmPH, *o*)	*Acinetobacter calcoaceticus* NCIB8250	CAA93242
Orf11/Phenol (anaerobic)	*Thauera aromatica*	CAC12684
PhcR/Phenol (LmPH, *m*)	*C. testosteroni* R5	BAA87867
PheR/Phenol (LmPH, *m*)	*P. putida* BH	BAA09883
PhhR/Methylphenols (LmPH, *m*)	*P. putida* 35X	CAA56111
PhlR/Phenol (LmPH, *m*)	*Ralstonia eutropha* JMP134	AAC77386
PhlR/Phenol (LmPH, *m*)	*P. putida* H (pPGH1)	CAA62584
PoxR/Phenol (LmPH, *m*)	*R. eutropha* E2	AAC32451
TbuT/Toluene and Phenols (ScPH, *m*)	*R. pickettii* PKO1	AAC44567
TouR/Toluene and Phenols (LmPH, *m*)	*P. stutzeri* OX1	CAB52211
GntR-family (repressors)		
AphS/Phenol (LmPH, *m*)	*C. testosteroni* TA441	
PhcS/Phenol (LmPH, *m*)	*C. testosteroni* R5	BAB61103
Arac/XylS family		
PhcT/Phenol (LmPH, *m*)	*C. testosteroni* R5	BAC01263
LysR family (activators)		
CatR/Phenol (ScPH, *o*)	*P. putida* (pEST1226)	AAA25768
AphT/Phenol (LmPH, *m*)	*C. testosteroni* R5	BAA88500

Representative regulators were chosen for their pertinence to the text.

[a]Note that, as described in the text, the pathways indicate initial substrate(s) that do not all necessarily serve as the effector compound of the cognate regulator. LmPH, large multicomponent phenol hydroxylase; ScPH, single component phenol hydroxylase. *m* and *o* indicate associated *meta*- and *ortho*-cleavage pathways for further catabolism, respectively.

[b]Accession designations are for the regulators of the indicated systems.

analogues possessing chloro- or ethyl-substituents. Tolerance of chemical variation is, however, markedly dependent on the nature and location of the substituents on the aromatic ring, being notably poor with compounds that harbour substituents in the *para*-position[71, 72]. The aromatic-responsive group of σ^{54}-dependent transcriptional regulators also includes members that are responsive to bi-aromatic compounds (e.g., HbpR)[33], and some that can respond to both mono-aromatic compounds and the non-aromatic pollutant trichloroethane (e.g., TbuT)[13]. Thus, this family of transcriptional activators has the capacity to respond to the whole range of effector molecules that can be metabolised or co-metabolised by the multicomponent phenol hydroxylases and similar proteins.

In spite of the broad effector specificity of some transcriptional activators, aromatic effector recognition by regulators can impose limitations on catabolic capacity through their poor or non-responsiveness to aromatic

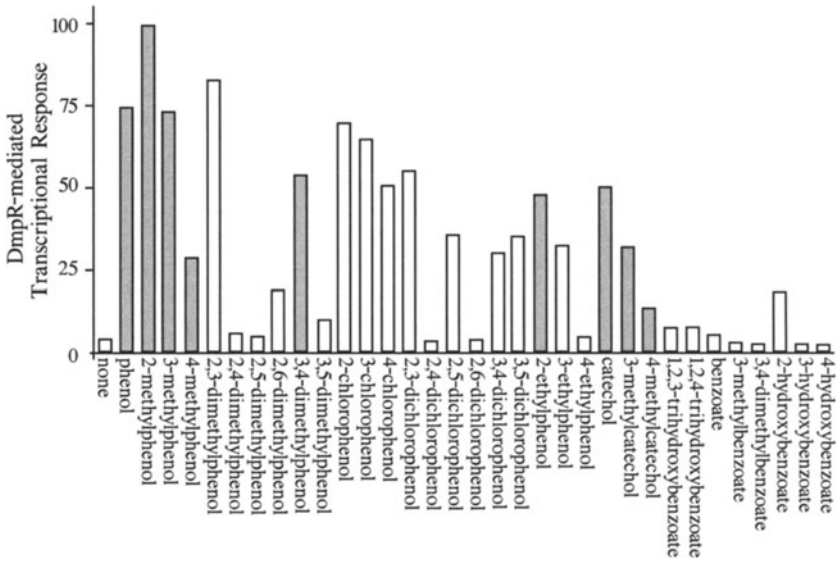

Figure 3. DmpR-dependent in vivo transcription in response to different aromatic compounds. The results are collated from *dmp*-operon promoter-luciferase reporter gene data using 0.1–2 mM effector reported in refs [52], [71], [72], and are presented relative to the maximum achieved with the best effector 2-methylphenol. DmpR is unresponsive to toluene, *o*-, *m*-, or *p*-xylene, 2-, 3-, or 4-methylbenzoate, benzyl alcohol, or 4-nitrophenol[66, 71]. Shaded boxes indicate compounds that serve as growth substrates via the *dmp*-encoded pathway of *Pseudomonas* sp. CF600.

compounds[63]. This has been demonstrated to be the case with both DmpR and the DmpR-like regulator PhhR. For *Pseudomonas* sp. CF600 harbouring the *dmp*-system of pVI150, poor recognition of 4-methylphenol by DmpR limits biodegradative capacity since it elicits sub-optimal expression of the enzymes necessary for its catabolism. Consistently, an effector specificity mutant that had removed the regulatory bottleneck by acquisition of an enhanced ability to respond to 4-methylphenol likewise enhanced catabolic performance[52, 66]. Similarly, poor recognition of 3,4-dimethylphenol by PhhR of *P. putida* P35X prevented growth at the expense of this compound, and simple introduction and expression of 3,4-dimethylphenol responsive DmpR that can cross-regulate the *phh*-operon promoter alleviated this limitation[42]. Hence, aromatic effector recognition by this family of transcriptional regulators can limit both the efficiency and substrate range of aromatic catabolism.

Many different strategies have been used to generate genetic diversity within the aromatic response profiles of σ^{54}-dependent regulators including genetic selections[22, 72], error prone PCR mutagenesis[90], DNA shuffling[29, 74], and in situ selection[66]. Structural variants with novel response properties were

primarily generated in order to dissect the molecular mechanism of aromatic-effector control, and to generate derivatives that recognise priority pollutants for incorporation as the biological component in whole cell biosensors. The ability to manipulate the response profile of these aromatic-responsive regulators illustrates the adaptability of this family of transcriptional activators. Furthermore, the plasticity of aromatic-responsiveness is not only observed in the laboratory. For *Pseudomonas* sp. CF600, as little as 2–4 days in 4-methylphenol contaminated soil results in a "fitter" mutant sub-population that can degrade 4-methylphenol more efficiently. This fitter phenotype was traced to the sole acquisition of aromatic-sensory mutations of the DmpR regulator, some of which had previously been identified during laboratory selections[66]. Hence, these are adaptive mutations that result in acquired enhanced fitness under prevailing conditions. Thus, selection of mutations that alter effector-regulatory responses to a given pollutant(s) is a continual and ongoing adaptation mechanism employed by microorganisms for optimising aromatic catabolism in contaminated environments. The distinct domain structure of aromatic-responsive DmpR-like proteins (see below) may facilitate fast adaptation of response profiles and be a contributing force resulting in the abundant use of this class of regulators in control of aromatic catabolic pathways.

3.2. From Effector Binding to Transcriptional Activation by Aromatic Responsive σ^{54}-Dependent Regulators

The σ^{54}-dependent family of transcriptional activators are unusual in that they are mechanoenzymes that utilise ATP hydrolysis to trigger remodelling of σ^{54}-RNA polymerase inter-subunit and DNA interactions to actively assist the DNA melt-step and thereby promote transcriptional initiation[91]. Regulators of this family exhibit a distinct domain structure and bear in common a central σ^{54}-activation domain that is also known as an EBP-module (for *e*nhancer-*b*inding *p*rotein) since these proteins typically act from binding sites located unusually distant from the promoters they control (see Figure 4A). The aromatic-responsive DmpR-like proteins form one of the subgroups within the σ^{54}-dependent family of transcriptional activators, which can be divided into different classes based on the mechanism by which the activities of the regulators are unlocked[70]. In addition to responding to small effector ligands, the activities of some members of this family are controlled by phosphorylation signal transduction cascades or by signal-responsive protein–protein interactions, all of which mediate control through the regulatory N-terminal A-domain that can harbour one or more sensory motifs[75].

For DmpR-like proteins, the sensory-A-domain is sufficient to directly bind the aromatic-effector and, based on competition assays employing different aromatic compounds and DmpR, a single site appears to be used for the multiple

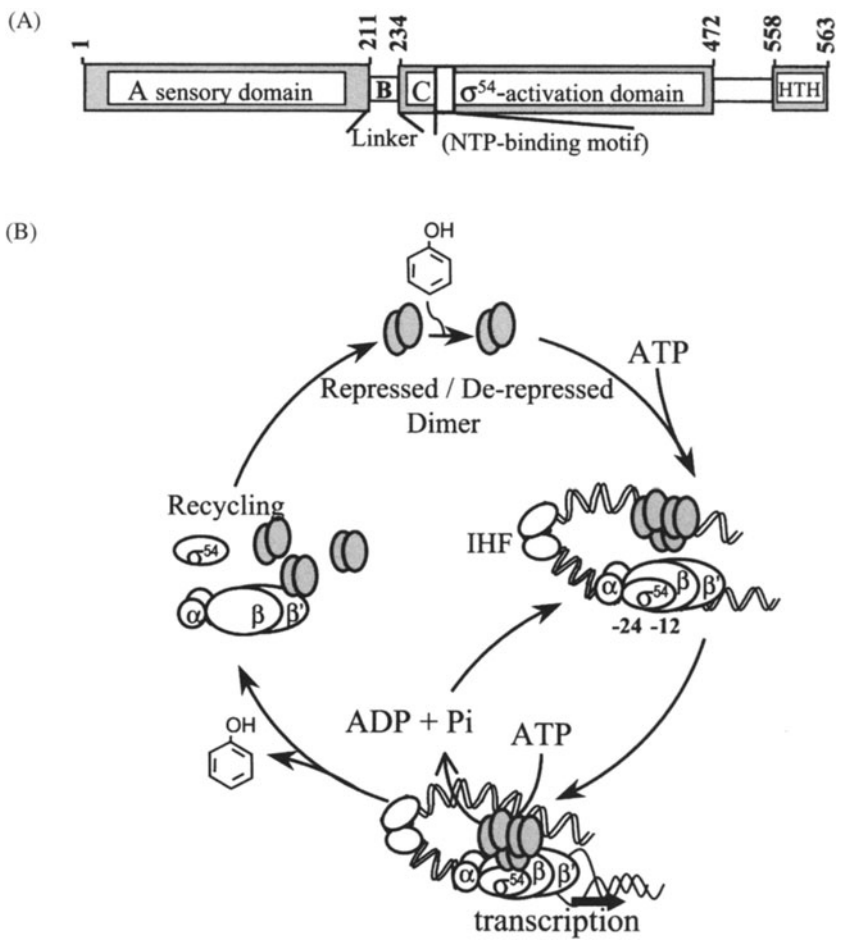

Figure 4. (A) Schematic illustration of the domain structure of σ^{54}-dependent regulators as exemplified by DmpR and detailed in the text. HTH stands for helix–turn–helix motif and indicates the domain responsible for DNA binding. (B) Model for the sequential steps identified during aromatic effector control of the transcriptional promoting activity of DmpR[88]. Binding of aromatic effectors to the sensory A-domain breaks repressive A/C-domain interactions and mediates a conformational change that allows an ATP-binding triggered dimer to hexamer transition. The active multimer, that is bound at a distance from the −24, −12 *dmp*-operon promoter is aided in its close physical association with holoenzyme RNA polymerase ($\alpha_2\beta\beta'\sigma^{54}$) by the DNA-bending protein IHF (integration host factor) and can then promote transcription as it undergoes hydrolysis of ATP. The cycling of transcriptional initiation and re-binding of ATP will continue only so long as the aromatic effector molecule is available. Upon loss of the effector signal and hydrolysis of ATP, the multimeric state of DmpR cannot be maintained and the components of the transcription complex will be recycled.

aromatic-effectors[49, 50]. Within these proteins the sensory A-domain serves to represses the innate transcriptional promoting property of the central σ^{54}-activation domain through specific inter-domain interactions that are broken upon aromatic-effector binding[25, 41, 53, 72]. Thus, truncated proteins that specifically lack the regulatory A-domain result in constitutively active regulators that are independent of aromatic-effectors for activity. A small structured-linker joins the sensory A-domain to the σ^{54}-interaction module in DmpR and many other aromatic-responsive members. This linker has been found to be directly involved in co-ordinating de-repression in response to aromatic-effector binding[28, 51]. Some mutations that alter and weaken the normally strong repressive inter-domain interaction lead to novel response properties[29, 41]. Mechanistically, gain of novel response properties does not necessarily involve creation of a new effector-binding property. Rather, where analysed in detail, the novel response property appears to lie within the ability of effector binding to productively couple to transcriptional activation by DmpR[50]. Thus, both aromatic-effector binding to the sensory A-domain and coordinated consequent release of inter-domain repression are required for transcriptional activation. The DNA binding activity mediated through the C-terminal domain has recently been shown to be coupled to and be stimulated by aromatic-effector binding in the family member HpbR[83]. However, ultimate control is determined by the ability of aromatic-effector binding to translate to a conformation that allows ATP-binding to trigger a multimeric state that is central for transcriptional activation *per se*[88] (Figure 4B). Intriguingly, the effector-bound form of the A-domain may also play an additional role in stabilising the active multimeric configuration of DmpR. Hence the sensory A-domain may play an active role in maintaining both the repressed and active state of this class of proteins[88].

Simple sequence comparisons of the sensory A-domains of aromatic-responsive σ^{54}-dependent regulators are insufficient to deduce the aromatic-effector profile as exemplified by MopR whose effector-response profile is most similar to DmpR but whose primary sequence is most similar to toluene/xylene responsive XylR[67]. A purely bioinformatics-based structural model for the sensory A-domains of this class of proteins has recently been developed. This model goes a long way to explain the distinct and non-overlapping response profiles of DmpR and XylR, and the experimental phenotypes observed with sensory A-domain mutations[23].

3.3. Strict Aromatic Effector Control vs Semi-Constitutive activity

The model outlined in Figure 4B can account for the exquisitely tight regulatory control observed with DmpR and XylR, since each transcriptional

initiation event is dependent on a sensing step to determine the presence of the aromatic-effector molecule. However, there is an increasing body of evidence that strict aromatic-effector control may not necessarily be the norm in all cases. As described below, phenol-responsive regulatory systems can employ members of this family that evidently incorporate a degree of aromatic-independent transcription into the regulatory strategy, in one case apparently by necessity, in another in combination with an additional regulatory device that serves to exert control.

An example of an apparent case-by-necessity comes from the *tou*-system of *P. stutzeri* OX1. The genetic organisation of this TouR regulated biodegradative system is illustrated in Figure 1B. The *tou*-system encodes the capacity to degrade toluene and *o*-xylene in addition to phenol and methylated phenols by virtue of two multicomponent monooxygenases, a toluene/*o*-xylene monooxygenase, and a multicomponent phenol hydroxylase, which have overlapping and redundant specificities[9]. The activity of TouR, rather than being responsive to toluene and xylenes, is directly controlled by phenols and methylated phenols. The response profile of TouR, together with the mosaic construction of the *tou*-gene cluster, strongly suggests that this catabolic unit has been expanded from a *dmp*-like operon by incorporation of the *touABCDEF* cluster as illustrated in Figure 1B[5]. As a consequence of such an event, toluenes and *o*-xylene would not be able to elicit gene expression of the enzymes for their own catabolism unless some degree of transcription occurred that allowed low basal level conversion to the TouR phenolic effector compounds. Low level effector-independent transcription from the TouR-regulated promoter of the toluene/*o*-xylene monooxygenase gene cluster has been detected by Northern analysis[6]. Thus it is possible that TouR possesses an intrinsic low level effector-independent activity in intact cells although this has not yet been observed in vitro[4].

A more extreme example comes from *C. testosteroni* R5 encoding the PhcR-dependent *phc*-system[81]. This strain bears similarities but also significant differences to *C. testosteroni* TA441 encoding the AphR-dependent *aph*-system[2] (see Figure 1B). Both these phenol catabolic systems, like the *dmp*-system of *Pseudomonas* sp. CF600, employ a multicomponent phenol hydroxylase, a subsequent *meta*-cleavage pathway, and an indispensable DmpR-like σ^{54}-dependent master regulator. However, both the *phc*- and *aph*-systems additionally employ a GntR-like repressor (PhcS/AphS) and a third unrelated additional regulator (AraC/XylS-like PhcT or LysR-like AphT). The phenol degradative system of *C. testosteroni* TA441 is apparently cryptic, being silent until the strain adapts to grow on phenol via inactivation of the GntR-like repressor AphS that binds to the *aphR–aphK* intervening region to block transcription[2]. Once adapted, the phenol responsive AphR regulator allows effector-responsive transcription of the *aphKLMOPQB* cluster that

encodes the enzymes for conversion of phenol to 2-hydroxymuconic semialdehyde. This intermediate is further catabolised through expression of the additional downstream *aph*-gene clusters, transcription of which is additionally stimulated by the 2-hydroxymuconic semialdehyde-responsive LysR-like AphT regulator[3] (see Figure 1B). *C. testosteroni* R5, on the other hand, is able to grow at the expense of phenol even when the GntR-like PhcS repressor is functional. In this case, inactivation of PhcS results in high level transcription of the cognate *phc*-gene cluster in the absence of the phenolic effector compound, giving enzyme activities equivalent to 66% of those observed when grown at the expense of phenol. Aromatic-effector control of this system appears to function through PhcS, but in a strain-specific fashion that is limited to *C. testosteroni* R5 indicating the involvement of an additional unknown factor[81]. A third regulator of this system, an AraC/XylS-type regulator PhcT (see Figure 1B) mediates an approximate 2 fold-enhancement of PhcR-induced enzyme levels, although the mechanism underlying the effects of PhcT remains to be resolved[82]. Despite the added complexity of this regulatory system, the key observation is that in the absence of PhcS, the σ^{54}-dependent PhcR mediates high levels of effector-independent transcription under conditions where the directly compared, tightly aromatic-effector controlled DmpR does not[81]. This property is strongly reminiscent of the semi-constitutive phenotype of DmpR mutants that possess single amino acid substitutions within the sensory-A domain, the central C-domain σ^{54}-interaction module, or the structured-linker that joins them[72]. These mutations weaken the repressive A/C-domain interaction and confer on DmpR the ability to promote some degree of transcription in the absence of an effector, while still maintaining effector responsiveness. Individual mutations that mediate between 16% and 42% of full effector activated levels can be combined to generate a derivative capable of promoting high effector-independent transcription equivalent to 63% of effector activated levels[41]. Thus, although it remains to be directly tested, it appears likely that aromatic-responsive σ^{54}-dependent regulators will differ in their degrees of effector-dependency through modulation of the strictness of the repressive A/C-domain interaction.

4. TRANSCRIPTIONAL INTEGRATION WITHIN THE HOST

The ability to catabolise aromatic compounds by peripheral pathways that feed into central metabolism is only advantageous to the host bacterium when preferred carbon sources are absent. Therefore, where studied, the transcriptional circuits of aromatic catabolic pathways have been found to be

integrated within, and subservient to, global regulatory systems that report on the nutritional and energetic state of the cell. A whole variety of different regulatory mechanisms have been identified that contribute to this end in specific cases. These include (a) competition of an effector-binding site of a regulator by the TCA-cycle intermediate fumarate (ClcR), (b) transcriptional inhibitory occupancy of a DNA-binding site under high pathway metabolic flow (CatR), (c) sensing through the activity of the electron transport chain (AlkS/PhlR), (d) environmental-responsive activity of a chaperon/protease (XylR), and (e) through the activity of a PTS-system (XylR) (reviewed in ref. [68], see Chapters 13 and 18). Thus only two additional mechanisms have been described below that have implications for transcription control of all σ^{54}-dependent systems; namely the recently proposed key role of (p)ppGpp in competition of σ^{54} for limiting core RNA polymerase, and the regulatory coupling mediated through the DNA-bending protein IHF (integration host factor).

4.1. Stress, (p)ppGpp, and σ-Factor Competition

Even in the presence of potent aromatic-effectors, the DmpR/Po σ^{54}-dependent system is effectively rendered silent during rapid growth on rich media until growth slows down at the transition to stationary phase. This transient phase crudely mimics "hungry" conditions under which bacteria globally respond to optimise scavenging capabilities. One such global response is the production of the alarmones ppGpp and pppGpp, collectively referred to as (p)ppGpp. These unusual nucleotides act through the β/β' interface of RNA polymerase and function to down-regulate excess translational capacity through decreased trancriptional output from the strong σ^{70}-P1 promoters of ribosomal RNA and protein operons during the stringent response[20]. The (p)ppGpp alarmones herald a multitude of stresses since they are synthesised by the ribosome-associated RelA and the dual functional SpoT protein in response to amino acid and nutrient (carbon, phosphate, or nitrogen) limitations, as well as physicochemical stresses. For the σ^{54}-dependent DmpR–Po regulatory system, restricted transcription under high-energy conditions provided by rich media and coupling to host physiology is primarily attributable to (p)ppGpp[79]. The transcriptional activity of this phenol-responsive regulatory circuit is restricted by low levels of (p)ppGpp that are elicited by rapid growth on rich media, and is only released to give high levels of transcription when (p)ppGpp levels are elevated at the exponential to stationary phase transition, or elevated due to growth at the expense of poor carbon sources[78, 79]. Consequently, transcription from the σ^{54}-dependent Po promoter is severely inhibited in both *Escherichia coli* and *P. putida* strains lacking RelA and SpoT, despite the constant levels of DmpR and σ^{54} in both strains[76]. Conversely, artificial elevation of (p)ppGpp levels under normally non-permissive high-energy conditions allows

DmpR–Po regulatory circuit to function even during exponential growth on rich media[79].

Decreased open-complex stability in the presence of (p)ppGpp has been proposed to underlie both its negative and positive direct effects on σ^{70}-promoters, leading to decreased transcription from stringent rrn promoters that intrinsically form very unstable open-complexes, and perhaps aiding promoter escape and thus increasing transcription from (p)ppGpp-stimulated σ^{70}-promoters[11, 92]. Addition of (p)ppGpp to in vitro transcription assays has only a relatively small direct stimulatory effect on the transcriptional output from the σ^{54}-Po promoter. The critical key to (p)ppGpp-dependent transcription from Po appears to lie not in a requirement for (p)ppGpp *per se*, but rather in the central role of (p)ppGpp in dictating the outcome of competition of σ^{54} for limiting core RNA polymerase[37]. Competition between the different σ-factors to form the alternative holoenzyme RNA polymerases required for promoter recognition and transcriptional initiation from the distinct class of promoters within the genome creates global hierarchies. Unlike σ^{S} and σ^{H} for which (p)ppGpp-dependent modulation of competition is critical[34], the levels of the structurally distinct σ^{54}, like those of σ^{70}, are constant throughout the growth curves of *E. coli* and *P. putida*[19, 32]. Using representative (p)ppGpp-stimulated σ^{S}-, σ^{H}-, and the σ^{54}-promoters, mutations that render σ^{70} less able to compete for core RNA polymerase in in vitro and in vivo assays were shown in all cases to restore transcription in strains unable to produce (p)ppGpp[34, 37]. Likewise, artificial mild underproduction or sequestering of σ^{70} via the anti-σ^{70} protein Rsd restores transcription in the absence of (p)ppGpp. In the case of the σ^{54}-dependent DmpR–Po circuit, σ^{70} underproduction also allows transcription during exponential growth on rich media, where (p)ppGpp levels are low and the Po promoter is normally silent[37].

The model for (p)ppGpp alteration of the relative competitiveness of σ^{54} and other alternative σ-factors proves a mechanism for global adjustment of the relative RNA polymerase holoenzyme pools to meet cellular demands during nutritional and physiological stress[34]. In addition, this mechanism would also amplify any direct stimulatory effects of (p)ppGpp observed at σ^{54}-Po and other promoters[37]. In this respect it is interesting that σ^{54} and σ^{70}, which exhibit similar high affinities for core when assessed in isolation, differ significantly when assessed under competitive conditions, with σ^{54} being markedly poorer at competing with σ^{70} than the converse in both in vivo and in vitro assays[37]. The levels of these proteins within the cell are also disproportionate. Taking available estimates from *E. coli*, ~110 molecules of σ^{54} are available to service ~30 potential σ^{54}-dependent promoters within the cell, while only 600–700 molecules of σ^{70} are available for >1,000 actively transcribed σ^{70}-dependent promoters[32]. The properties of constant comparatively high levels and high affinity of σ^{54}, together with (p)ppGpp-stimulation of its otherwise poor competitive ability against σ^{70}, provides a mechanism for rapid alteration in

occupancy and transcription from σ^{54}-dependent promoters in response to environmental changes without *de novo* σ^{54} synthesis.

The σ^{54}-protein was first investigated through its role in transcriptional regulation of genes involved in nitrogen assimilation and fixation. Since then many physiological processes that are responsive to environmental cues have been found to utilise σ^{54}. In addition to the involvement of σ^{54} in the control of *P. putida* carbon catabolism through catabolism of many aromatic compounds, σ^{54} is also involved in controlling many carbon catabolic systems in *P. aeruginosa*[44]. Thus, (p)ppGpp modulation of the levels of the available σ^{54}-RNA polymerase pool has the potential to globally affect the transcriptional output from different σ^{54} promoters and have far reaching affects on many integrated physiological processes. However, the model does not exclude that the low pool of σ^{54}-RNA polymerase available during high-energy conditions and rapid growth is sufficient to occupy and initiate transcription from certain high affinity σ^{54}-promoters. Thus, the extent to which (p)ppGpp-modulation of σ^{54}-RNA polymerase levels is manifested at different σ^{54}-promoters is predicted to depend on their innate or assisted ability to recruit the holoenzyme[37], although this remains to be directly tested.

4.2. (p)ppGpp, the IHF Link, and a Common Phenotype by Different Means

As described above, the (p)ppGpp-signalling molecule can have direct effects on promoter kinetics of specific promoters. As a consequence of its stimulatory effects on promoters that control other global regulators, (p)ppGpp is an accessory and amplifying factor in several global regulatory cascades. For example, the levels of IHF are partially under the control of (p)ppGpp in *E. coli*, and expression of IHF is up-regulated upon entry into stationary phase in both *E. coli* and *P. putida*[8, 85]. This site-specific DNA-bending protein impacts many promoter types and processes and is by itself a global regulator[7]. IHF is required by many σ^{54}-dependent promoters to assist close physical proximity and thus interaction between the distally bound regulators and the promoter-bound σ^{54}-RNA polymerase (see Figure 4B). However IHF plays additional roles at σ^{54}-dependent promoters, including (a) restricting transcriptional activation to a single regulator or signal transduction pathway, (b) aiding binding of the regulator, (c) stimulation of open-complex formation and/or stability, and (d) assisting σ^{54}-RNA polymerase recruitment (see ref. [77] and references therein).

Optimal transcriptional output from both the DmpR–Po and XylR–Pu σ^{54}-dependent systems requires IHF. At the Po promoter, IHF stimulates open-complex formation and/or stability by an as yet unresolved mechanism, and

transcription in IHF null *E. coli* and *P. putida* is reduced by approximately 75%[77, 79]. The Pu promoter is much more dependent of IHF and transcription in IHF null *P. putida* is barely detectable[16]. At the Pu promoter, IHF plays a major role in recruitment of σ^{54}-RNA polymerase by providing an architecture that allows interaction of the α-subunit of RNA polymerase with a distally located "UP-like" DNA element that is otherwise out of contact[12, 17]. The in vivo occupation of the IHF site of Pu in response to the changing levels of IHF plays a key role in restricting the capacity of Pu promoter during exponential growth, although additional signals are also incorporated at the exponential to stationary phase transition[85]. This recruitment role for IHF is not observed for the Po promoter, nor has it yet been reported for any other σ^{54}-dependent promoter. Thus it is currently unknown how extensively distributed this σ^{54}-RNA polymerase recruiting devise will turn out to be.

Triggering of the production of (p)ppGpp upon nutrient depletion appears to play a key role at both the Po and Pu promoters, but by different apparent mechanisms[76]. In a side-by-side comparison, the stimulatory effect of (p)ppGpp is markedly higher on Po than the Pu promoter in vitro[18]. These direct effects, observed in the absence of σ-factor competition, would be amplified in vivo by the (p)ppGpp-mediated superimposed regulation of available pools of σ^{54}-RNA polymerase as described under Section 4.1. Consistently, comparison of the impact of the absence of (p)ppGpp in vivo using (p)ppGpp null *E. coli* and *P. putida* strains showed that the XylR–Pu system was markedly less affected than the DmpR–Po system[76]. Thus, for the Po promoter available σ^{54}-RNA polymerase levels appear of primary regulatory importance. For the Pu promoter, IHF occupancy is a strict requirement for transcriptional output, and low levels of IHF during exponential growth and consequent lack of recruitment of the available σ^{54}-RNA polymerase would be of primary importance in limiting transcription. However, as described above, this limitation would be alleviated in a partial (p)ppGpp-dependent manner as the IHF levels increase as the cells cease growing exponentially, at a similar point as where available pools of σ^{54}-RNA polymerase increase. Thus, for the two very similar aromatic-responsive DmpR–Po and XylR–Pu pairs, differing direct effects of (p)ppGpp on promoter kinetics, (p)ppGpp regulation of available σ^{54}-RNA polymerase pools, and (p)ppGpp-dependent IHF levels, probably differentially combine to lock the functioning of both of these promoters to the conditions encountered at the exponential to stationary phase transition.

5. CONCLUDING REMARKS

Direct sensing of aromatic substrates or their metabolites by transcriptional regulators permits substrate detection and provides the specific response

for expression of cognate aromatic catabolic pathways. As underscored in this chapter, the response profile of regulators can evidently limit the catabolic potential of the bacteria. Regulators of the aromatic-responsive σ^{54}-dependent family of transcriptional activators appear particularly adaptable in their effector-response profiles. The generally broad response profiles and adaptability to allow a response to structural aromatic variants through minor genetic changes probably contributes to the predominant use of this class of regulators in controlling pathways for aromatic compounds, in particular, for those that comprise broad specificity enzyme systems. The molecular mechanisms that allow binding of aromatic compounds to unlock the transcriptional promoting activities of σ^{54}-dependent activators are relatively well understood. However, key questions such as the potential active role of the effector-bound sensory domain in maintaining the active form, and how the active form engages the σ^{54}-RNA polymerase to promote transcription are still not fully resolved.

In contrast, the multitude of global regulatory mechanisms that influence the expression of aromatic compounds in response to environmental clues are still mostly poorly understood. As exemplified by the DmpR–Po and XylR–Pu regulatory circuits, even mechanistically very similar systems can be integrated by disparate means. Once again, the flexibility in how the activity of σ^{54}-dependent activators can be simply and effectively subdued by global regulatory systems may also contribute to the frequent adoption of this class of regulators in controlling pathways for catabolism of aromatics that feed into central metabolism. To date, all the global regulatory systems that are known to impact aromatic catabolic systems in Pseudomonads mediate control at the level of transcription[68]. In most cases, information on the underlying molecular mechanism and/or sensing and signal transduction mechanisms are largely missing and severely limit our understanding of the complete picture. Moreover, small non-coding RNAs that exert control on the translational level have been increasingly implicated in housekeeping and stress responses[84] and in coordinating adaptation processes in *E. coli*[64]. Such small regulatory RNAs have been found to influence σ^{54}-dependent systems[1] and to be involved in the control of carbon flow in *E. coli*[54]. Analogous systems are undoubtedly utilised in global regulation in Pseudomonads, thus this class of regulatory molecule is likely to feature more prominently in our understanding of coordinated metabolic coupling in the years to come.

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ENZYMATIC ACTIVATION OF THE *cis–trans* ISOMERASE AND TRANSCRIPTIONAL REGULATION OF EFFLUX PUMPS IN SOLVENT TOLERANCE IN *PSEUDOMONAS PUTIDA*

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1. INTRODUCTION

Organic solvents with a log P_{ow} (logarithm of its partition coefficient in *n*-octanol and water) between 1.5 and 4.0 are extremely toxic for microorganisms and other living cells because they partition preferentially in the cytoplasmic membrane, disorganizing its structure and impairing vital functions[99].

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The toxicity of these compounds depends not only on the inherent toxicity of the solvent but also on the intrinsic tolerance of the bacterial species and strains. The level of organic solvent tolerance in *Escherichia coli* is variable among strains; that is, *E. coli* JA300 and MC1061 grow in the presence of *n*-hexane (log P_{ow} 3.9) whereas *E. coli* MV1184 or DH1 do not[1, 2], and it has been proved to be enhanced by mutations. Most *Pseudomonas* species are highly sensitive to aromatic hydrocarbons such as toluene (log P_{ow} 2.5), styrene (log P_{ow} 3.05) and *p*-xylene (log P_{ow} 3.2); however, independent laboratories have isolated *Pseudomonas putida* strains tolerant to these toxic compounds[10, 37, 52, 80, 106].

What then makes one specific strain more tolerant than another? Several elements have been suggested to be involved in the response to these toxic chemicals. Some responses, such as rigidification of the cell membrane via alteration of the phospholipid composition or alteration of the cell surface to make the cells less permeable try to prevent the entrance of toxic compounds into the cells[27, 29, 43]. Among the changes in phospholipid composition, *cis/trans* isomerization of unsaturated fatty acids and changes in the saturated-to-unsaturated fatty acid ratio are considered to be the short- and long-term response to solvent exposure, respectively[47]. The increase in *trans* isomers (which are directly synthesized from the *cis* isomers with no shift in the position of the double bond) as well as an increase in saturated fatty acids, cause rigidification of the membrane to counteract the increase in membrane fluidity caused by the organic solvent[28, 31, 59]. Although changes in phospholipid fatty acids are not essential for solvent tolerance, they probably represent a first response that allows the cells to gain time for the *de novo* biosynthesis of other components involved in tolerance toward organic solvents. One such response was thought to be the metabolism of the organic solvents, but some of the tolerant strains are not able to degrade the compound. For example, *P. putida* S12 is a toluene-tolerant strain but it cannot degrade this solvent[107], and a *P. putida* DOT-T1E mutant deficient in the *tod* (toluene-dioxygenase) degradation pathway is as tolerant as the wild type to a sudden toluene shock[64]. These two observations suggest that degradation of the toxic compound is not a key factor in solvent tolerance. Some active processes of elimination of the compounds are also implicated in tolerance, for example, the formation of vesicles that remove solvents from the cell surface and the extrusion of organic solvents by efflux pumps[53, 83, 84]. Among these mechanisms, the efflux pumps are the primary cause of solvent tolerance in several *P. putida* strains.

Bacteria have been exposed during evolution to different natural toxic compounds (toxins, secondary metabolites, antibiotics, etc.) which have made them to develop different strategies to detoxify or eliminate these compounds. One such group of mechanisms comprises the multidrug resistance efflux systems, which catalyze the active extrusion of many structurally and functionally

unrelated compounds from the bacterial cytoplasm (or inner part of the cytoplasmic membrane) to the external medium[74, 88]. Surprisingly, some of the substrates of these efflux pumps are xenobiotics that do not resemble any of the known natural compounds that these cells may have encountered during evolution. All the efflux pumps for organic solvents identified so far in gram-negative bacteria belong to the RND family. The functioning of these efflux pumps seems to be coupled to the proton motive-force via the TonB system, although the intimate mechanism of energy transfer remains elusive[20, 116]. Bacterial RND efflux pumps work together with a membrane fusion protein (MFP) and an outer membrane protein (OMP). These three components form a structure that traverses both the inner and outer membranes[56, 66, 113]. All the sequences of RND efflux pumps are predicted to have 12 transmembrane domains and two large periplasmic loops between the transmembrane segments TMS1 and TMS2 and between TMS7 and TMS8. The efflux pump transporter AcrB of the *E. coli* RND multidrug efflux system AcrAB-TolC was recently crystallized. The AcrB component exists as a trimer with a 50-Å transmembrane region and a 70-Å part located in the periplasm that is thought to be involved in substrate recognition[66, 111]. The crystal structure of the OMP TolC—which forms a trimeric channel that penetrates the periplasm and contacts the efflux pump transporter—has also been reported[56]. Finally, a lipoprotein anchored to the inner membrane which expands into the periplasmic space may serve as a bracket for the other two components[113, 114]. This structural organization allows substrates to be extruded into the external medium[17, 56, 66]. The detailed structure of the RND systems is described elsewhere in this book[78].

2. CHANGES IN MEMBRANE FATTY ACID COMPOSITION IN RESPONSE TO ORGANIC SOLVENTS

Already in the first paper dealing with solvent-tolerant bacteria[37], changes in the membrane composition were predicted to play a crucial role in the mechanisms contributing to solvent tolerance because the membrane is the main target of the toxic action of the organic solvents[29, 99]. The toxicity of the organic solvents is caused by general physico-chemical effects and neither metabolic nor chemical reactions are associated with the toxic effects; the accumulation of the organic solvent in the membranes leads to a non-specific permeabilization[9, 26, 30] that compromises the membrane function as a barrier, as matrix for enzymes and as energy transducer[98, 107].

The fluidity of the cell membrane is affected not only by organic solvents but also by other environmental stresses (i.e., increases in temperature)

and several adaptive responses at the membrane level have been described including changes in lipid composition and in membrane proteins[39, 44, 45, 51, 84, 95, 107]. With these changes, cells try to keep the fluidity of their membranes in the same shape regardless of the environmental conditions. This stabilization of the membrane fluidity is known as "homeoviscous adaptation."[100] The main adaptive mechanisms at the level of membrane fatty acids described so far in *Pseudomonas* cells are: (a) the degree of saturation of the fatty acids, (b) *cis/trans* isomerization of unsaturated fatty acids and (c) cyclopropanation of bacterial membrane lipids.

Cyclopropane fatty acids are formed during the stationary phase from *cis*-unsaturated fatty acids[9, 21, 93] and its biosynthesis is known to be regulated by the RpoS sigma factor[6, 16] in *E. coli*. Cyclopropane fatty acid synthesis is increased by certain changes in environmental conditions in *Pseudomonas denitrificans*[40, 41], and *E. coli* strains defective in the cyclopropane fatty acids synthase are more sensitive to killing by a rapid shift from neutral pH to pH 3 (see ref. [5]). Recently, it has been shown that overexpression of the cyclopropane fatty acid synthase increases butanol resistance in *Clostridium acetobutylicum* ATCC824[117], but the role of cyclopropanation in *Pseudomonas* solvent tolerance remains unclear.

It is well known that some bacterial cells react to the presence of organic solvents by increasing the amount of saturated fatty acids in the membrane[8, 36, 44, 46, 77]. Alterations in the saturation degree of the fatty acids change the fluidity of the membrane and in this way compensate the effects caused by solvents. Phospholipids containing 16:0 saturated fatty acids have a transition temperature which is about 63°C higher than those containing 16:1*cis* unsaturated fatty acids[8, 23]. *Pseudomonas* cells, like most bacteria, synthesize fatty acids by the well-established pathway which has been studied in detail in *E. coli*. This pathway is described elsewhere in this book[93]. One major disadvantage of changes in the degree of saturation as the major adaptive mechanism lies in the strong dependency of this reaction on cell growth and fatty acid biosynthesis. It has been observed that organic solvents cause a shift in the ratio saturated to unsaturated fatty acids only up to concentrations that do not completely inhibit cell growth (Figure 1). In the presence of higher concentrations of the toxic compound the cells cannot react in this way and are therefore not able to adapt to these conditions or even die[8, 30].

Most species of the genus *Pseudomonas*, are able to quickly convert their *cis* into the corresponding *trans* unsaturated fatty acids after exposure to toxic compounds[27, 47, 81]. Contrary to the previously described modification of the degree of saturation, this conversion also takes place in non-growing cells and when the biosynthesis of fatty acids was inhibited by cerulenin[8, 12, 28]. Figure 1 shows the differences between the two mechanisms against increasing concentrations of a toxic compound such as toluene; the degree of saturation

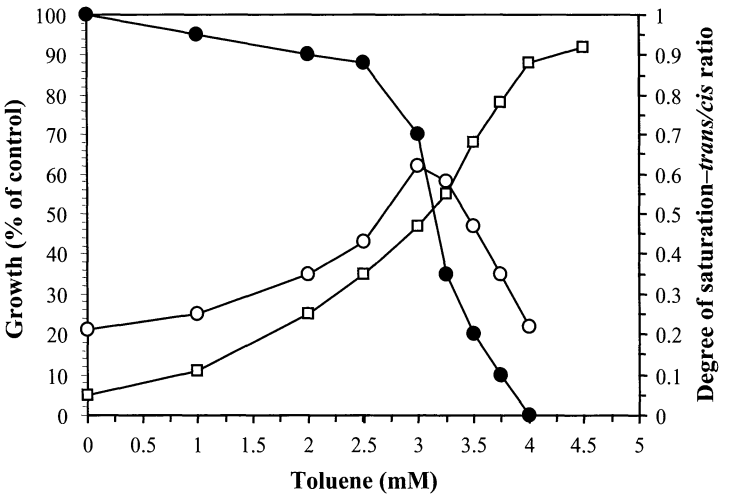

Figure 1. Effect of toluene on several parameters. Toluene was added to non-adapted *P. putida* cells in the exponential growth phase at 30°C. Three hours after toluene addition, the cells were harvested to determine, bacterial growth (●), degree of saturation of membrane fatty acids (○), and *trans/cis* ratio of unsaturated fatty acids (□).

reaches a certain maximum at a toluene concentration that causes a growth inhibition of about 50% while the *trans/cis* ratio of unsaturated fatty acids still increases at toluene concentrations that completely inhibited cell growth. The isomerization of the double bond is a special mechanism to adapt to high concentrations of toxic compounds under conditions not allowing growth and *de novo* synthesis of lipids[70]. The benefit of this reaction lies in the steric differences between *cis* and *trans* unsaturated fatty acids. Unsaturated fatty acids in the *cis*-configuration with their bended steric structures (a nick in the acyl-chain) result in a membrane with a relatively high fluidity. In contrast, the long extended structure of the *trans*-configuration is able to insert into the membrane structures similarly to saturated fatty acids[47, 62].

2.1. The *cis/trans* Isomerase

The *cis–trans* isomerase (Cti) is a neutral protein of 87,000 kDa that is constitutively expressed, it does not require ATP or other cofactors like NAD(P)H or glutathione, and works in the absence of *de novo* synthesis of lipids[12, 27, 31]. Its independence from ATP agrees with the negative free energy of the *cis*-to-*trans* isomerization[97].

The *cti* gene was cloned and sequenced from *P. putida* P8, *P. putida* DOT-T1E and *Pseudomonas* sp. E-3[34, 43, 71]. Alignment studies have proved

that Cti is highly conserved and only exhibits minor differences among *Pseudomonas* strains[105]. The deduced amino acid sequences revealed that the isomerase has an N-terminal hydrophobic signal sequence, which should be cleaved off after targeting the enzyme to the periplasmic space. In fact, the enzyme has been purified from the periplasmic fraction of *Pseudomonas oleovorans* and *Pseudomonas* sp. strain E-3 and was isolated as a his-tagged *P. putida* P8 protein heterologously expressed in *E. coli*[34, 71, 75].

Holtwick and coworkers provided the first indication that the enzyme is a cytochrome *c*-type protein and they found a heme-binding site in the predicted Cti polypeptide[35]. Comparison of the amino acid sequences of the seven (up to the present) known Cti-proteins, identified them as heme-containing proteins of the cytochrome *c*-type[35, 105]. For an enzyme preparation from *Pseudomonas* sp. strain E-3, which is presumably homologous to the *cti*-gene product of *P. putida* P8, iron (probably Fe^{3+}) was suggested to play a crucial role in the catalytic reaction[71]. The mechanism of *cis/trans* isomerization does not include transient saturation of the double bond[105].

The Cti protein is independent from the cardiolipin synthase, as a knockout mutant in this enzyme is still able to isomerize *cis* unsaturated fatty acids[104]. Cardiolipin synthase is involved in the long-term adaptation of the membrane to organic solvents by increasing cardiolipin synthesis[81, 104, 107].

The *cis–trans* isomerization of unsaturated fatty acids was detected 5 min after the addition of the organic solvent, which suggests that it is a very fast mechanism that is triggered to overcome organic solvent toxicity. To prove that *cis/trans* isomerization is necessary for the survival of solvent-tolerant strains, studies were performed with mutants lacking the ability to perform this isomerization. A transposon mutant of the solvent-tolerant *P. putida* DOT-T1 was both solvent-sensitive and unable to perform the isomerization[81]. Furthermore, a Cti knockout mutant was constructed in *P. putida* DOT-T1E. This mutant was slightly less resistant to the 0.08% (vol/vol) toluene shock than the wild-type strain and showed a longer lag phase when grown with toluene (in the gas phase) as the only carbon source[43]. This data suggested that although the *cis/trans* isomerization is unlikely to be the only adaptation mechanism to organic solvents, it is probably the first barrier that the cells interpose to cope with these chemicals.

2.2. Regulation of the *cis–trans* Isomerase

One of the main questions that remains open regarding the Cti of unsaturated fatty acids in *Pseudomonas* (and several *Vibrio*) strains is how the activity of this constitutively expressed periplasmic enzyme is regulated. Several stress factors such as organic solvents, osmotic stress caused by NaCl and sucrose, heavy metals, heat shock and membrane-active antibiotics were

shown to be activators of the Cti system[13, 25, 27, 31, 38]. Only those stress conditions that do not activate K^+-uptake (the first cellular reaction to membrane damage leading to increased permeabilization[11]) such as osmotic stress caused by glycerol, cold shock and high pH, caused no activation of the Cti[31]. This indicates that the *cis/trans* ratio may be part of the microorganisms general stress-response[4, 31].

The question is: how can these environmental factors activate Cti activity? One of the possibilities could be a complex model in which the substrate of the enzyme, the *cis*-unsaturated fatty acids, are cleaved from the periplasmic phase of the membrane phospholipids. The resulting free unsaturated fatty acids would then be isomerized by Cti action and subsequently reattached to the lysophospholipid, resulting in phospholipid containing *trans*-unsaturated fatty acids[9]. This very complex model is not in agreement with data that confirm Cti activity in resting cells and in the complete absence of energy sources, as at least the reattachment of the modified fatty acids to the lysophospholipids would need energy[27].

Another possibility could be that the activation of enzyme activity may be brought about by giving the active center of the enzyme the ability to reach its substrate, the double bond, which in turn could depend on the fluidity state of the membrane. Accordingly, the observed regio-specificity reflects penetration of the active site of the isomerase to a specific depth in the membrane[7]. The hydrophilic structure and periplasmic location of Cti supports the assumption that the enzyme can only reach the targets that are located at a certain depth in the membrane, when the membrane is "opened" in the presence of environmental conditions that lead to its disintegration[32]. It has been previously shown that a decrease in acyl chain order can result in increased penetration and translocation of proteins in membranes[51]. Analogous to the known behavior of certain phospholipases, it is possible that the Cti could display increased penetration of the membrane when the order of the acyl chains is decreased and the spacing of the phospholipid head groups is increased. It is also possible that the decreased packing of the membrane could allow the double bonds to approach the membrane surface more frequently[32], facilitating the interaction with the isomerase[7]. As the acyl chain package is increased by *cis* to *trans* isomerization of the unsaturated fatty acids[94], protein penetration would be counteracted and *cis*-to-*trans* isomerization would be inhibited. This would allow the acyl chain package to be tightly regulated without the involvement of indirect signaling mechanisms. Therefore, this model for the regulation of the Cti activity also explains the often described relation between the intensity of *cis–trans* isomerization with toxicity caused by a certain concentration of an environmental stress factor[30, 31].

After removal of the solvent, recovery of the regularly very low *trans–cis* ratio is most likely done by the normal *de novo* synthesis of all-*cis* fatty acids, because a reverse (*trans*-to-*cis*) process would require an energy input.

3. TRANSCRIPTIONAL REGULATION OF THE ORGANIC SOLVENT EFFLUX PUMPS IN *PSEUDOMONAS PUTIDA*

The energy-dependent efflux pumps belonging to the resistance-nodulation-cell division (RND) family, which export toxic organic solvents to the external medium, have been proved to be one of the most important factors in solvent tolerance[48, 52, 57, 60, 82, 110]. In a recent study, several *P. putida* strains were analyzed with regard to toluene tolerance[96]. Three of these strains have been classified as highly resistant (*P. putida* DOT-T1E, *P. putida* S12 and *P. putida* MTB6). In *P. putida* DOT-T1E, three efflux pumps are involved in solvent tolerance: TtgABC[82], TtgDEF[65] and TtgGHI[85]. The same three efflux pump operons are present in the *P. putida* MTB6 chromosome although their participation in organic solvent extrusion has not been studied in detail. *P. putida* S12 contains two of these efflux pumps encoded by the *arpABC* genes (98% identical to *ttgABC*)[50], and the *srpABC* (99% identical to *ttgGHI*), although only one of these efflux pumps, SrpABC, has been implicated in solvent tolerance in the S12 strain[48]. In *P. putida* DOT-T1E and S12, the efflux pumps involved in solvent tolerance have been extensively characterized although the study of their genetic regulation is still in progress.

In strains *P. putida* DOT-T1E and S12, solvent tolerance is an inducible process. For example, growth of *P. putida* DOT-T1E in the presence of toluene supplied in the gas phase has a clear effect on cell survival: the sudden addition of 0.3% (vol/vol) toluene to *P. putida* DOT-T1E pre-grown with toluene in the gas phase resulted in survival of almost 100% of the initial cell number, whereas only 0.01% of the cells pre-grown in the absence of toluene, tolerated exposure to this aromatic hydrocarbon[81, 82] (Figure 2A). The three efflux pumps in this strain should therefore work together to achieve the maximal level of solvent tolerance, and they are probably tightly regulated in order to produce an optimal response to solvent stress.

Most of the regulatory genes that encode for proteins involved in control of the expression of the efflux pumps belonging to the RND family are located adjacent to the structural genes of the pump, and generally the regulatory gene is transcribed divergently from the efflux pump operon. Some of these regulatory proteins are transcriptional activators such as MexT (positive regulator of the MexEF-OprN efflux pump of *Pseudomonas aeruginosa*[55]), but most of them act as repressors, for example, MexR (negative regulator of the MexAB-OprM pump of *P. aeruginosa*), AcrR (regulator of the AcrAB-TolC efflux pump of *E. coli*) and SmeT (that is the regulator of the SmeDEF pump of *Stenotrophomonas maltophilia*)[61, 89, 101]. In this chapter we will review, using as models the three efflux systems in *P. putida* DOT-T1E, the regulation of the efflux pumps involved in solvent tolerance in the two highly tolerant strains,

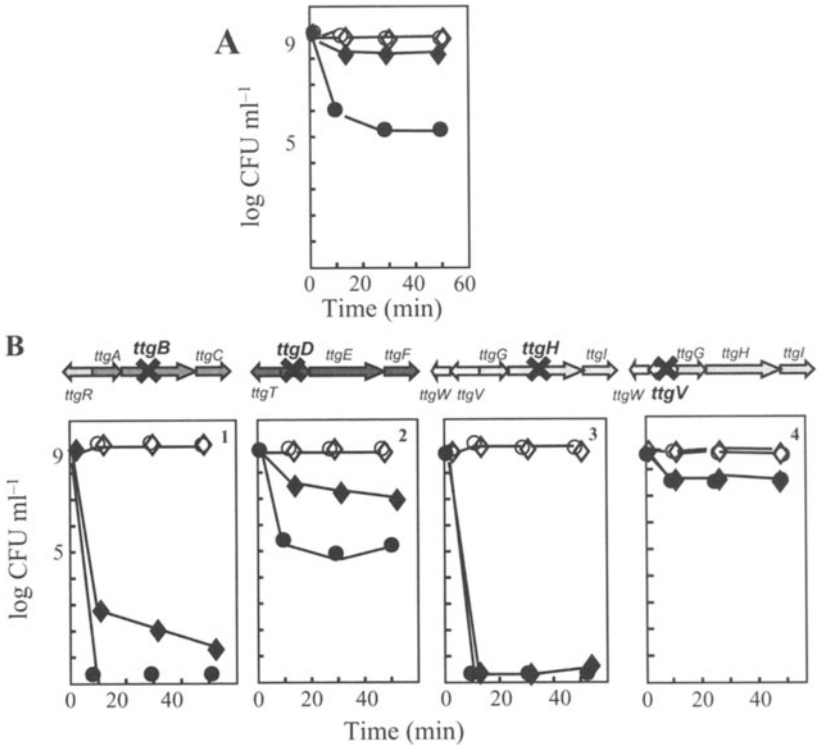

Figure 2. Survival of *P. putida* DOT-T1E wild type and mutant strains upon toluene shock. Cells were grown in 30 ml of LB (circles) or LB with toluene in the gas phase (diamonds) until the culture reached a turbidity of 0.8 at 660 nm. The cultures were divided in two halves; to one 0.3% (vol/vol) toluene was added (close symbols) and the other was kept as a control (open symbols). The number of viable cells was determined before toluene addition and 10, 30 and 60 min later. A. *P. putida* DOT-T1E; B. 1. *P. putida* DOT-T1E-18 (*ttgB::'phoA*-Km); 2. *P. putida* DOT-T1E-1 (*ttgD::kilAB*); 3. *P. putida* DOT-T1E-28 (*ttgH::*ΩSm); 4. *P. putida* DOT-T1E-62 (*ttgV::aphA3*). The arrows indicate the efflux pump gene organization and the corresponding regulatory protein/s in each system. The specific gene mutated in each efflux pump is crossed.

P. putida DOT-T1E and *P. putida* S12, and we will discuss the possible "global regulation" of the efflux pumps.

3.1. Regulation of the *ttgABC* Efflux Pump Operon

The TtgABC efflux pump was the first efflux pump identified in *P. putida* DOT-T1E as involved in solvent tolerance. Physiological experiments done with a *ttgB* knockout mutant suggested that this efflux pump was involved in the so-called intrinsic tolerance. This mutant (*P. putida* DOT-T1E-18) did not withstand

the sudden toluene shock (0.3% vol/vol) at all, and only a small but significant fraction (about 1 out of 10^5 cells) survived the shock if pre-exposed to low toluene concentrations (Figure 2B.1)[82]. On the basis of this observation, the existence of other efflux pump(s) involved in toluene extrusion was postulated. The fact that in *P. putida* DOT-T1E-18 cultures, no survival at all was observed after sudden toluene shock compared with the 0.01% cell survival observed in the wild type pointed out to a high basal expression of the TtgABC pump responsible for this noninduced intrinsic resistance. This hypothesis was confirmed with primer extension analysis to study the expression levels of the *ttgABC* operon. In fact, the operon is transcribed at relatively high levels in the presence and also in the absence of toluene in accordance with its physiological behavior[15, 103].

The TtgABC efflux pump was previously shown to be involved in the multidrug resistance phenotype of *P. putida* DOT-T1E: the *ttgB* knockout mutant was significantly less resistant than the wild type to different antibiotics such as ampicillin, carbenicillin, tetracycline, nalidixic acid and chloramphenicol[82]. Therefore, the TtgABC efflux pump was able to extrude not only different organic solvents such as toluene, styrene or *p*-xylene, but was also able to accommodate different antibiotics[15, 82, 85] (Table 1). In fact, the broad substrate specificity of MDR efflux pumps is the main feature responsible for bacterial multiple resistance to very different and unrelated antimicrobial agents. In *P. aeruginosa*, three of the Mex efflux pumps responsible for multiple antibiotic resistance (MexAB-OprM, MexCD-OprJ and MexEF-OprN) have been shown to contribute to organic solvent tolerance of this organism[57]. The AcrAB-TolC and AcrEF-TolC efflux pumps that extrude fluoroquinolones and other antibiotics are also important for *n*-hexane tolerance in *E. coli*[1, 54].

Gene fusion to *lacZ* and primer extension assays showed that some substrates of the TtgABC efflux pump, such as chloramphenicol or tetracycline, did increase (to a different extent) the expression of the pump operon, whereas others (nalidixic acid, streptomycin or carbenicillin) did not[103] (Table 1). Therefore, the contribution of this solvent efflux pump to antibiotic resistance should be regarded as due to its broad substrate specificity and in some cases, also to the induction of the efflux pump genes in response to certain antimicrobial agents.

Adjacent to the genes encoding the efflux pump and upstream of the *ttgABC* operon, there is an open reading frame (named *ttgR*) that encodes for a protein that shares 50–60% sequence similarity with a number of transcriptional repressors such as AcrR (repressor of the *acrAB* operon in *E. coli*) or MtrR (regulator of the MtrCDE efflux pump in *Neisseria gonorrhoeae*[61, 72]). All these proteins belong to the TetR family of transcriptional regulators[33]. TtgR is a repressor of the TtgABC efflux pump: a *ttgR* knockout mutant (*P. putida* DOT-T1E-13) exhibited an increased expression (6-fold higher than the wild type) of the efflux pump operon. However, this increase in the efflux pump transcription levels did not lead to an increase in the survival rate of the

Table 1. Multidrug efflux substrates (S) or inductors (I) of the efflux pumps.

	TtgABC		TtgDEF		TtgGHI		SrpABC		ArpABC	
	S	I	S	I	S	I	S	I	S	I
Toluene	+	−	+	+	+	+	+	+	−	n.d.
Styrene	+	+	+	+	+	+	n.d.	+	n.d.	n.d.
Propylbenzene	+	−	−	+	+	+	n.d.	+	n.d.	n.d.
m-xylene	+	−	−	+	+	+	n.d.	n.d	n.d.	n.d.
p-xylene	n.d.	n.d.	n.d.	n.d.	n.d.	+	+	+	−	−
Ethylbenzene	+	±	−	+	+	+	+	+	n.d.	n.d.
Hexane	n.d.	−	n.d.	n.d.	n.d.	−	+	+	n.d.	−
Heptane	n.d.	n.d.	n.d.	n.d.	n.d.	−	n.d.	+	n.d.	n.d
1-pentanol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	n.d.	n.d.
1-hexanol	n.d.	n.d.	n.d.	n.d.	n.d.	+	n.d.	+	n.d.	n.d.
1-octanol	+	n.d.	+	n.d.	+	+	+	+	n.d.	n.d.
Chloramphenicol	+	+	−	−	±	−	n.d.	±	+	−
Tetracycline	+	+	−	−	±	−	n.d.	±	+	n.d.
Nalidixic acid	+	−	−	−	±	−	n.d.	n.d	+	n.d.
Ampicillin	+	n.d.	−	n.d.	±	−	n.d.	±	+	n.d.
Carbenicillin	+	−	−	−	±	−	n.d.	n.d.	+	−
Streptomycin	+	−	n.d.	−	n.d.	n.d.	n.d.	−	+	n.d.
Erythromycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−	+	−
Novobiocin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	−	+	n.d.

+, The chemical is a substrate or effector of the efflux pump; −, The chemical is not a substrate/effector of the efflux pump; ±, The compound is a poor substrate/effector of the efflux pump; n.d. not determined.

culture when shocked with 0.3% (vol/vol) toluene, but did increase resistance toward different antibiotics such as chloramphenicol, carbenicillin and tetracycline[15]. In this mutant strain (*ttgR*$^-$), the basal activity of the *ttgR* promoter was 8-fold higher than the wild-type background, indicating that TtgR also down regulates its own transcription.

ttgR gene expression was very similar to that observed for its cognate efflux pump operon: both are induced by some organic solvents, and more significantly by chloramphenicol and tetracycline[15, 103] (Table 1). This clear similarity in the expression pattern of both promoters was unexpected, as TtgR was shown to be a repressor, and increased expression of a repressor would lead to greater transcriptional repression and not to induction, as was observed. In fact, induction could be only achieved if the agents that are able to induce expression from both *ttgABC* and *ttgR* promoters were able to modulate the TtgR repressing function exerted on these promoters.

The transcription initiation points of the *ttgA* and *ttgR* operons were mapped. The location of the start sites indicates that the divergent promoter regions fully overlap (Figure 3A). The −10 region, although not the −35 region,

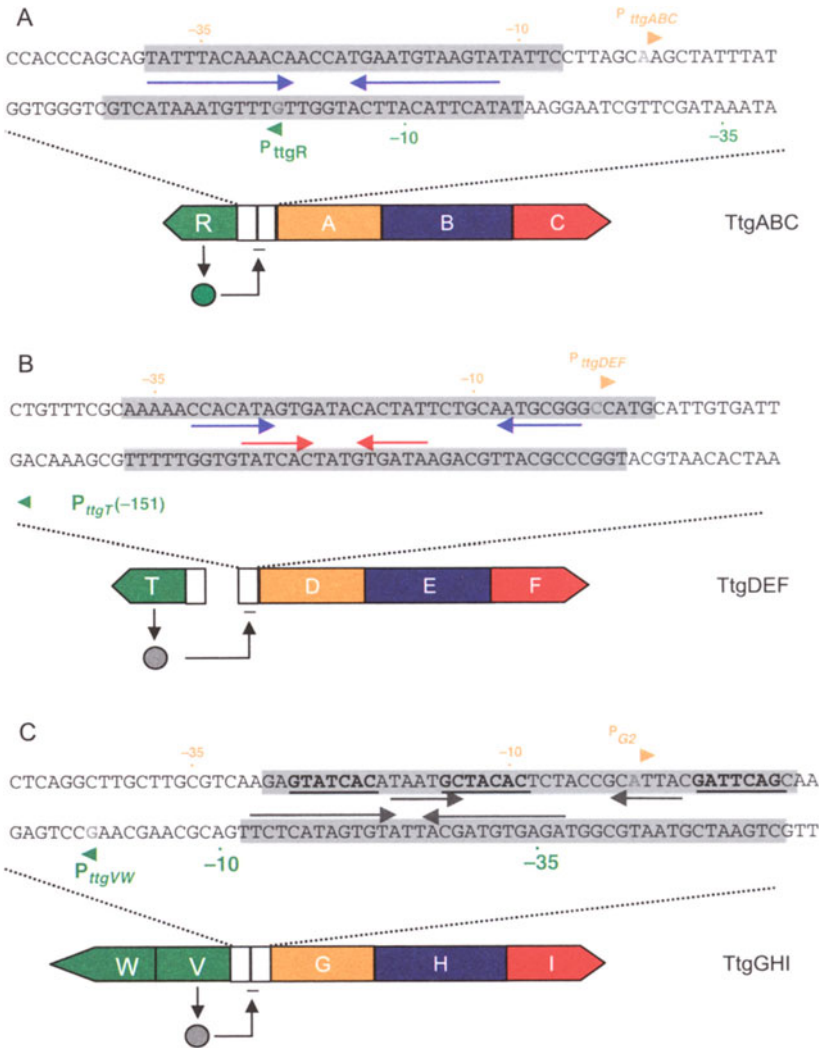

Figure 3. Organization of the A. *ttgABC*, B. *ttgDEF* and C. *ttgGHI* operons and their respective regulatory genes. The regulatory regions of each gene cluster are zoomed. A. TtgR, B. TtgT and C. TtgV DNA binding regions, deduced from DNAseI footprinting, are shadowed. Putative palindromic (arrows) or symmetric (bold and underlined) recognition sites for each repressor are indicated. The +1 and the direction of transcription are marked with small triangles for each promoter (except for *ttgT* one, which distance from P_{ttgDEF} is indicated). The −10 and the −35 positions of each promoter are also shown.

of both *ttgA* and *ttgR* promoters exhibits some degree of similarity to promoters recognized by sigma 70[15]. Although the common regulatory elements and the overlap of the two promoters explains some of the great similarity in their expression pattern, only the identification of the TtgR repressor operator site will explain the apparent co-regulation of these divergent genes.

A recombinant and functional His-tagged TtgR protein has been overexpressed, purified and used in several in vitro experiments designated to elucidate its regulatory role in *ttgABC* and *ttgR* expression. Gel shift assays showed that TtgR binds specifically to a DNA fragment corresponding to the *ttgR–ttgABC* intergenic region. Terán and coworkers (2003) demonstrated that the addition of chloramphenicol or tetracycline to the binding reaction led to a gradual dissociation of TtgR from the *ttgR–ttgABC* intergenic region, suggesting that TtgR is able to bind these two antibiotics, which triggers its release from the promoter regions with subsequent enhanced expression.

DNAse I footprint assays were carried out in the absence and presence of structurally unrelated antibiotic substrates of the TtgABC pump which allowed the identification of the TtgR operator within the *ttgA–ttgR* intergenic region and confirmed that dissociation of the operator and regulator was mediated by the antibiotic. When the binding reaction was performed in the absence of antibiotic, TtgR protected from DNAse I digestion a 36-bp DNA segment that includes the −10 and −35 region of the *ttgABC* promoter and the −10 of the *ttgR* promoter (Figure 3A). This protection was lost in the presence of chloramphenicol or tetracycline, confirming that in the presence of these antibiotics, TtgR dissociates from its operator site. The presence of several other antibiotics which had shown no inducing effect on *ttgABC/ttgR* gene expression (nalidixic acid, carbenicillin or streptomycin) did not cause TtgR dissociation[103]. This was the first evidence of the direct involvement of TtgR in the drug-dependent enhancement of *ttgABC* expression. The TtgR binding site, deduced from DNAse I footprint, revealed a particularly long inverted repeat (IR) (28 bp) comprising two 12-bp half sites separated by 4 bp. This indicates that probably each half site would accept two monomers of TtgR, as reported for the TtgR homolog QacR (repressor of the *qacA* multidrug pump gene of *Staphylococcus aureus*), whose 3D crystal structure bound to its operator was resolved[91].

Therefore, the molecular mechanism of TtgR transcriptional regulation has become more clear: the N-terminal DNA binding domain, which is highly conserved among members of the TetR family of regulators, possesses a helix-turn-helix (HTH) motif that forms the DNA sequence "reading" head of the protein and allows TtgR binding to its operator. This domain is connected to the C-terminal domain which is probably the ligand binding site that would receive structurally different effectors such as chloramphenicol or tetracycline. This ligand binding triggers a conformational change in the repressor that

probably provokes its dissociation from the operator, allowing RNA polymerase transcription from both promoters. Given that TtgR binds structurally dissimilar compounds as tetracycline and chloramphenicol and these drugs are both hydrophobic molecules, it will not be surprising that TtgR could also bind the organic solvents that increase *ttgABC* expression.

The broad substrate/ligand specificity of the efflux pumps and their corresponding regulatory proteins is apparently a contradiction of some of the basic dogmas of biochemistry[69]. In general, multidrug transporters may efflux many hydrophobic compounds, but not every hydrophobic drug can be extruded by these pumps. The molecular basis of multidrug recognition is not yet fully understood but the elucidation of the crystal structures of the proteins bound to their substrates/ligands will help to clarify the polyspecificity of these proteins. In AcrB, it has been shown that three molecules of ligands bind simultaneously to the large central cavity of the protein, primarily by hydrophobic, aromatic stacking and Van der Waals interactions. Each ligand uses a slightly different subset of AcrB residues for binding[111]. The structure of the QacR also revealed the presence of at least two different but overlapping drug binding pockets of aromatic nature[90–92]. These binding pockets have also been described in BmrR (bacterial multidrug resistance regulator of *Bacillus subtilis*[118]). Furthermore, in this regulatory protein, the flexibility of one of the binding site walls allows ligands of different structures to adapt their shape within the binding pocket to attain a maximum complementarity to the surrounding hydrophobic residues. It is probable that all these multidrug transporters and regulatory proteins contain multiple drug binding "mini-pockets" within a larger pocket[92].

In *P. putida* S12, the ArpABC efflux pump showed an overall identity of 99% at the amino acid level with the TtgABC of *P. putida* DOT-T1E. However, the ArpABC efflux pump has been reported not to be involved in solvent tolerance but in antibiotic resistance[50]. Upstream of the structural genes, there is an open reading frame that encodes for a protein (ArpR) 98% identical to the TtgR protein. Although its function has not been studied, it is quite possible that this protein is involved in the regulation of the ArpABC efflux pump. The *mepABC* efflux pump of a toluene-resistant variant of *P. putida* KT2442 has also been implicated in solvent and antibiotic resistance, and its sequence is practically identical to those of the TtgABC and ArpABC efflux pumps. The corresponding regulatory protein is probably encoded by *mepR*, although its function as a regulator has not been investigated yet[19].

Although TtgABC-like efflux pumps are widespread in different *P. putida* strains (*P. putida* MTB5, *P. putida* KT2440, *P. putida* SMO116, among others) with different levels of toluene tolerance[96], there are no studies of the role of these efflux pumps in solvent or antibiotic resistance, and research is lacking on the regulation of this pump in those strains.

3.2. Regulation of the *ttgDEF* Efflux Pump Operon

As described above, solvent tolerance studies in a *ttgB* knockout mutant of *P. putida* DOT-T1E (*P. putida* DOT-T1E-18) have suggested the existence of other inducible solvent efflux pump(s). By sequencing downstream from the toluene dioxygenase (*tod*) operon of *P. putida* DOT-T1E, Mosqueda and Ramos (2000) identified three open reading frames (*ttgDEF*) that encode for the three components of an efflux pump which shares homology with other efflux pumps of *Pseudomonas*. The transporter, named TtgE, shares 59% identity with the previously described TtgB and 75% identity with TtgH. The contribution of this efflux pump to solvent tolerance was studied in a *ttgD* knockout mutant (*P. putida* DOT-T1E-1). A culture of this mutant strain had a survival rate identical to the wild type when shocked with 0.3% (vol/vol) toluene. However, if the cells were pre-induced with toluene in the gas phase, the survival rate of the mutant was 100 times lower than in the wild type (Figure 2B.2). Expression studies of the *ttgDEF* operon at the transcriptional level revealed that this pump is not expressed during growth under normal laboratory conditions, and demonstrated its inducible character in the presence of organic solvents (toluene or styrene) (see ref. [65]; Table 1).

The wild-type multiple antibiotic resistance was not affected in a TtgDEF-deficient strain; moreover, no increase in antibiotic resistance was obtained by pre-inducing the culture with toluene[65], suggesting that the substrate specificity of this pump is limited to organic solvents. There was also no induction of the *ttgDEF* operon in the presence of several antibiotics in the culture media (Terán *et al.*, unpublished; Table 1).

Upstream from the *ttgDEF* operon and divergently transcribed, there is an open reading frame whose product shares homology with several members of the IclR family of transcriptional regulators[102]. This gene, called *ttgT*, encodes for a protein 70% identical to the SrpS-negative regulator of SrpABC solvent efflux pump of *P. putida* S12 (see below).

A *ttgT* knockout mutant showed a small increase in *ttgDEF* expression (1.5-fold) under noninducing conditions, suggesting its involvement in the negative regulation of this operon. The fact that in this mutant strain there was still strong induction of the *ttgDEF* efflux pump in the presence of organic solvents suggested that TtgT is not involved in the induction of this operon by organic solvents. These two lines of evidence pointed to the involvement of another still unknown regulator in the inducible response of this pump (Terán *et al.*, unpublished). Basal expression from the *ttgT* promoter was higher than the expression of its cognate efflux pump and remained constant regardless of the growth conditions and the inducers tested (organic solvents or antibiotics) in both wild type and TtgT-deficient strains. The identification of the transcription initiation point of both *ttgDEF* and *ttgT* operons[65], (Terán *et al.*, unpublished) revealed

that they are separated by 150 bp, a distance that allows independent transcriptional regulation of these two divergent promoters with subsequent differences in the expression of both operons (Figure 3B). These results differ from those observed for other regulators of efflux pumps with the same gene organization, in which negative autoregulation and similar expression patterns of both regulatory and structural operons seemed to be a common characteristic.

The repression exerted by the TtgT protein in the *ttgDEF* promoter was confirmed by reconstitution of the regulatory system in a heterologous host (*E. coli* CC118). The receptor strain carries the *ttgDEF* promoter in a plasmid-born transcriptional fusion to *lacZ* and the *ttgT* gene in *trans* under the control of a constitutive promoter (P_{lac}) in different-copy-number plasmids. As expected, the activity of P_{ttgDEF} significantly decreased when the copy number of the plasmid bearing the *ttgT* gene increased (Terán *et al.* unpublished). TtgT binding to the *ttgDEF* promoter was confirmed in vitro using a recombinant His-tagged TtgT protein. Gel shift experiments showed that TtgT was able to specifically bind a DNA fragment containing the *ttgT–ttgDEF* intergenic region (Terán *et al.*, unpublished). Moreover, DNAse I footprint assays revealed a single binding site along the *ttgT–ttgDEF* intergenic region which covers only the *ttgDEF* promoter region (−37 to +5 from the transcription start point) and not the *ttgT* one, consistent with the in vivo expression studies described above. Therefore, TtgT is directly involved in *ttgDEF* operon repression, probably by competing with the RNA polymerase for access to the efflux pump promoter. The protected area deduced from DNAse I footprint defines a large 42-bp operator site for the TtgT repressor (Figure 3B) (Terán *et al.*, unpublished). However, analysis of the operator sequence does not reveal the presence of a clear single IR, as reported for other members of the IclR family (TM-IclR of *Thermotoga maritima*[115], IclR of *E. coli*[14, 73]). Only short and imperfect 6-bp IR or direct repeats can be predicted within the operator sequence, suggesting a different sequence recognition mechanism for this protein. Further in vitro studies are needed to understand TtgT DNA binding mode and *ttgDEF* efflux pump operon regulation. Furthermore, the induction of this efflux pump by organic solvents remains unexplained as it has been suggested that another unknown regulator different from TtgT is involved in this efflux pump regulation. This theory deserves our attention regarding solvent efflux pump regulation because it leads to the idea that these *Pseudomonas* solvent efflux pumps may be regulated at a higher level, as observed in other bacterial species (see below, see also refs [3], [67], [79]).

3.3. Regulation of the *ttgGHI* Efflux Pump Operon

The third efflux pump involved in solvent tolerance in *P. putida* DOT-T1E is called TtgGHI. In a solvent tolerance analysis of different *P. putida*

strains, it was found that only the three highly toluene-tolerant strains of the study (*P. putida* DOT-T1E, S12 and MTB6) hybridized against a *ttgH* probe. This finding suggested that the TtgGHI-like efflux pumps are a key factor in the high level of resistance to toluene in several *P. putida* strains[96]. A TtgGHI-like efflux pump has also been identified in the toluene-resistant bacterium *P. putida* GM73[52]. Only two TtgGHI-like efflux pumps have been studied and characterized so far: the TtgGHI system of *P. putida* DOT-T1E and the SrpABC efflux pump of *P. putida* S12. A knockout mutant in which the TtgGHI efflux pump of *P. putida* DOT-T1E is not functional (*P. putida* DOT-T1E-PS28) is not able to survive a sudden 0.3% (vol/vol) toluene shock regardless of the growth conditions (Figure 2B.3)[85]. In contrast, the TtgABC and TtgDEF knockout mutants were still able to survive the toluene shocks to a different extent (Figure 2B.1 and 2). The extreme toluene sensitivity of the *P. putida* DOT-T1E-PS28 mutant strain (*ttgH::*ΩSm) under induced or noninduced conditions suggested that this efflux pump is involved in intrinsic as well as inducible resistance to organic solvents. The *ttgGHI* operon is 99% identical at the nucleotide level to the *srpABC* genes of the highly solvent-tolerant strain *P. putida* S12. A transposon insertion mutant in this operon lost the solvent-tolerant phenotype of *P. putida* S12, leading to the conclusion that this efflux pump is also one of the main factors in this strain's solvent tolerance[48].

It has been demonstrated that both the *ttgGHI* and the *srpABC* efflux pumps are induced by solvents[49, 85] (Table 1). The *srp* promoter of *P. putida* S12 was cloned into the *lacZ* reporter plasmid pKRZ-1 and the transcriptional fusion transformed in the parental strain, then β-galactosidase levels were determined under different conditions. Compounds such as aromatic and aliphatic solvents and alcohols (toluene, styrene, *p*-xylene, pentane, hexane, 1-pentanol, 1-heptanol and others) induced *srpABC* operon transcription. β-galactosidase levels increased with increasing concentrations of toluene; at 1 mM toluene, there was a 3.5-fold increase in expression and at 6 mM a 16.9-fold increase. There was only a 2-fold induction of the *srp* operon in the presence of 128 μg/mL chloramphenicol, 128 μg/mL ampicillin and 4 μg/mL tetracycline. General environmental factors such as different pH, low temperature, high levels of NaCl or the presence of organic acids did not induce *srp* transcription[49]. Similar results have been obtained in studies of *ttgGHI* expression in the *P. putida* DOT-T1E strain. The pattern of expression of the *ttgGHI* genes in the wild-type cells grown on LB medium with or without toluene supplied via the gas phase was determined by primer extension analysis. The *ttgGHI* operon was reported to be expressed from two overlapping promoters (P_{G1} and P_{G2}) at a certain basal level in the absence of solvents, and its expression increases from both promoters several-fold in the presence of aromatic hydrocarbons[85], although later it was demonstrated the expression from a single promoter, P_{G2}, renamed as P_G (see below). Fusion of the *ttgGHI*

promoter region (P_{ttgG}) to the promoterless *lacZ* gene in the low- copy-number pMP220 vector confirmed the results obtained by the primer extension analysis: β-galactosidase levels increased in the presence of organic solvents such as toluene or styrene but not in the presence of antibiotics such as chloramphenicol, tetracycline and others[86]. The TtgGHI efflux pump was shown to extrude antibiotics, although the effect of this transport was only detectable when the TtgABC efflux pump was not active[85] (Table 1). All these data showed that the expression patterns of the efflux pumps in both strains are very similar.

In both *P. putida* S12 and DOT-T1E, two genes (named *srpS* and *srpR* in *P. putida* S12 and *ttgV* and *ttgW* in *P. putida* DOT-T1E) were identified upstream from the corresponding efflux pump operons. These genes are transcribed divergently from the corresponding *srpABC* and *ttgGHI* operons. SrpS and TtgV showed 97% identity in their amino acid sequences. Both proteins showed an overall 50–60% similarity with a number of transcriptional regulators belonging to the IclR family[102]. *srpR* and *ttgW* showed 96% identity at their nucleotide level but *srpR* encoded a 213-amino-acid protein, whereas *ttgW* encoded a 134-amino-acid protein. The nucleotide sequence beyond the stop codon of *ttgW* in *P. putida* DOT-T1E remains 96% identical to the sequence of the *srpR* gene. These two proteins showed similarity with members of the TetR family of repressors[33], although as mentioned above, TtgW seemed to be a truncated member of the family. RT-PCR assays have demonstrated that *ttgV* and *ttgW* formed an operon[86]. Although this has not been demonstrated in *P. putida* S12, the two ORFs encoded by *srpS* and *srpR* genes are separated only by 5-bp and both are probably part of the same transcriptional unit.

A knockout mutant of the TtgV protein has been constructed and characterized[86]. The mutant strain (*P. putida* DOT-T1E-PS62) showed increased resistance toward toluene shocks under noninduced conditions when compared with the wild type. The fraction of cells that survived the sudden addition of 0.3% (vol/vol) toluene was the same (10^7 cells) under induced and noninduced conditions (Figure 2B.4). Analysis of the expression of the *ttgGHI* operon in this genetic background showed that the level of transcription increased 4-fold in the absence of toluene. Taken together, these data clearly indicated that TtgV is a repressor that prevents expression of the *ttgGHI* operon. A knockout mutant in which the *ttgW* gene was interrupted by an Ω-Km interposon showed the same level of resistance as the wild type, and the expression pattern of *ttgGHI* was also similar to the wild type. A double mutant (TtgV and TtgW deficient) was as resistant to the solvent shock as the single mutant in *ttgV*, and the expression levels of the efflux pump operon were also similar to those in the *P. putida* DOT-T1E-PS62 mutant. These results suggested that at least under the conditions assayed, TtgW did not play a significant role in the regulation of the

ttgGHI operon in *P. putida* DOT-T1E probably because this strain produces a truncated form of the regulatory protein.

The transcription initiation point of the *ttgVW* operon was mapped in cells growing in the absence and in the presence of toluene. The operon was transcribed from a single promoter regardless of the growth conditions, but the level of expression in the presence of toluene was 3- to 4-fold higher than in the absence of the aromatic hydrocarbon. The *ttgVW* operon was also shown to be induced by several organic solvents but not by antibiotics. The *ttgVW* operon showed a pattern of inducibility similar to that of *ttgGHI*, probably because both promoters are regulated in the same way. Sequence analysis of the promoter region showed that the −10 and −35 boxes of the P_{G2} overlap with the −35 and −10 boxes of the *ttgV* promoter (Figure 3C). β-galactosidase assays carried out with a transcriptional fusion of P_{ttgV} promoter to *lacZ* in a TtgV-deficient background showed that expression of the *ttgVW* promoter was about 3-fold higher than in the wild-type strain, also in the absence of toluene, indicating that TtgV negatively controls its own expression. Expression of P_{ttgV} in the *ttgW*-ΩKm background was similar to the wild type and in the TtgV-TtgW-deficient background it was similar to P_{ttgV} expression in the *P. putida* PS62 mutant. Together, these results suggested that the TtgV protein is a repressor of its own synthesis as well as of *ttgGHI* operon expression[86].

The TtgV protein has been overexpressed and purified with an N-terminal histidine tag. In vitro gel mobility shift assays were done to demonstrate the specific binding of the regulatory protein TtgV to a 210-bp DNA fragment between the *ttgG* and *ttgV* genes. Sequence analysis of P_{ttgG} and P_{ttgV} suggested that both promoters overlapped, and DNAse I-footprint assays were carried out to define the region in which TtgV binds within the *ttgV–ttgG* intergenic region. The DNAse I-protected region contained a 40-bp region that covers the −10/−35 regions of the *ttgG* promoter and the divergently oriented *ttgV* promoter[86] (Figure 3C). To gain insight into the mechanism of regulation of *ttgGHI* transcription by TtgV, in vitro transcription experiments were done using the purified protein and a construction of the supercoiled DNA plasmid pTE103 ligated to the *ttgV–ttgG* intergenic region. When the TtgV protein and the plasmid were incubated before the addition of RNA-polymerase, *ttgGHI* transcription was completely repressed. However, when TtgV was added after the formation of the RNA–polymerase–*ttgGHI* promoter open complex, the repression level became negligible (Guazzaroni *et al.*, unpublished). These findings support the concept that TtgV binding to the intergenic region blocks the entry of RNA–polymerase to transcribe these genes[87]. In these in vitro transcription experiments, only one transcriptional unit was revealed which corresponds with the presence of only one promoter region (P_{G2}) instead of the two promoters (P_{G1} and P_{G2}) proposed before. Several promoter mutant sequences were generated by direct mutagenesis, by changing a single base of

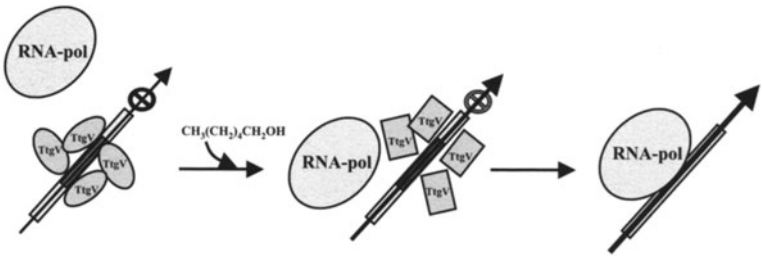

Figure 4. Schematic and highly hypothetical representation of TtgV-DNA binding process, based on the proposed model of the IclR protein. A. TtgV tetramer will bind to the target DNA; B. binding of 1-hexanol (inducer) to the TtgV protein will disrupt the tetramer, decreasing their affinity for DNA; C. RNA polymerase will bind to the DNA promoter region increasing the transcription.

the putative −10 boxes of the two proposed *ttgG* promoters in each mutant sequence. These sequences were fused to the *lacZ* gene in the pMP220 plasmid and β-galactosidase activity was measured in the absence and in the presence of toluene in the wild-type strain carrying the corresponding plasmids. β-galactosidase assays confirmed the results of the in vitro transcription experiment, ruling out the possibility of two overlapping promoters for the *ttgGHI* operon (Guazzaroni *et al.*, unpublished).

In vitro transcription assays were carried out in the absence and in the presence of increasing concentrations of a known inducer of *ttgGHI* operon expression. Addition of the inducer (1-hexanol) to the P_{ttgG} transcription reaction mixture in the presence of TtgV led to transcriptional levels similar to those observed in the absence of TtgV repressor, resulting in derepression in vitro. This suggests that 1-hexanol decreased TtgV binding to the intergenic region (Figure 4). This reduction in DNA binding correlates with recovery of the basal transcription level of the *ttgGHI* operon (Guazzaroni *et al.*, unpublished).

Although such a detailed genetic analysis of the regulation of the *srpABC* operon of *P. putida* S12 by the SrpS and SrpR proteins has not been achieved, some aspects of the regulation of these homologous efflux pumps seem to be quite different between the two strains (*P. putida* DOT-T1E and *P. putida* S12). In *P. putida* S12, an insertion sequence (IS*S12*) plays a key role in toluene tolerance[108]. Preadaptation to toluene in *P. putida* S12 endows the cells with the ability to survive a second phase of the solvent (1% vol/vol). Without preadaptation, most of the cells in the culture died. However, a few cells in the population were able to survive the sudden addition of 1% (vol/vol) toluene, and these cells were able to maintain their toluene-tolerant phenotype, suggesting a transition into a solvent-tolerant genetic variant. This phenotype has not been observed in *P. putida* DOT-T1E. The IS*S12* sequence was initially isolated from a chloramphenicol-resistant mutant that carries a 2.5-kb

interruption in the gene coding for the SrpC element of the efflux pump. Under normal culture conditions, the *P. putida* S12 genome contained seven copies of this IS element; however, a population growing to high cell density after the sudden addition of a separate phase of toluene carried eight copies. The extra element was located in the *srpS* gene. The basal level of *srp* promoter activity in this *P. putida* S12 "variant" (with eight IS copies in the chromosome) was seven times as high as in the original strain and frequency of survival to the sudden addition of toluene to this culture was 1,000 times as high as in the "normal" *P. putida* S12 cells. These results indicated that SrpS, like its homologous TtgV in *P. putida* DOT-T1E, is a negative regulator of the efflux pump *srpABC*. Complementation of the variant cells with the *srpSR* genes showed a dramatic decrease in toluene tolerance, but complementation with *srpS* alone did not result in decreased tolerance, suggesting that both SrpS and SrpR are needed for effective repression of the *srpABC* operon[108]. No data are available for the levels of expression of the *srpSR* genes in these "variant" cells.

In *P. putida* DOT-T1E, all efforts to identify an IS element similar to IS*S12* have failed. PCR amplifications were run using primers based on the sequence of IS*S12* and DNA of cells grown on LB medium or LB plus 0.3% (vol/vol) toluene as a template, but no amplification product was obtained. Southern blot analysis with an IS*S12* probe of *P. putida* DOT-T1E chromosomal DNA extracted from cells grown under different conditions also failed to reveal hybridization bands. These findings indicate that no element similar to the IS*S12* was present in the *P. putida* DOT-T1E genome despite the high sequence homology between different genes of these two strains (Segura *et al.*, unpublished).

Insertion of the IS186 sequence in the *acrR* gene that encodes for the repressor protein of the *acrAB* efflux pump in *E. coli* also resulted in increased expression of the corresponding efflux pump. Furthermore, it was also demonstrated that another insertion (IS2) in the promoter region of *acrE* also increased the expression of the *acrEF* efflux genes. In these two cases, the increase in expression of the efflux pumps led to increased resistance to fluoroquinolones[42]. The data from *E. coli* and *P. putida* S12 suggested a common regulatory process by which the insertion of an IS element into a repressor gene or in the promoter region of the efflux operon increased the tolerance to different toxic substrates by overexpression of the efflux pump responsible for their elimination. The insertion of IS elements other than IS*S12* of *P. putida* S12 was also investigated; PCR experiments using *P. putida* DOT-T1E chromosomal DNA isolated from cells grown to high density with a second phase of toluene did not reveal any insertion within the *ttgVW* operon or the *ttgV–ttgG* intergenic region (Segura *et al.*, unpublished). Since no evidence of insertions within the regulatory genes or the intergenic region was

detected in *P. putida* DOT-T1E, the conclusion is that this IS-mediated up regulation of efflux pumps is probably not involved in TtgGHI-mediated solvent tolerance in this strain.

The findings reviewed above indicate that solvent-tolerance in the two solvent-tolerant strains *P. putida* DOT-T1E and *P. putida* S12 shares some characteristics, but the systems also have interesting differences, as in the role of TtgW/SrpR proteins and the presence or absence of an IS element in the regulation of *srpABC/ttgGHI* operons.

3.4. Cross-Regulation of the Efflux Pumps

TtgT and TtgV proteins share a high degree of sequence identity (63%) and belong to the same family of regulatory proteins (IclR). *ttgDEF* and *ttgGHI* expression is induced by the same chemicals: organic solvents, but not antibiotics (Table 1). Furthermore, sequence alignment of *ttgDEF* and *ttgGHI* promoter regions revealed a high degree of identity (Figure 3B, C). This implies either cross-regulation involving their known local regulators, or possible co-regulation at different levels.

In vivo expression experiments showed that in a TtgV-deficient background, the basal and solvent-induced levels of *ttgDEF* were enhanced (Terán *et al.*, unpublished). This result may reflect the involvement of the TtgV regulator in *ttgDEF* repression. In vitro experiments demonstrated that TtgV binds specifically to the *ttgDEF–ttgT* intergenic region with similar affinity to that of TtgT. Although such clear evidence of the role of TtgT in *ttgGHI* repression has not been obtained from in vivo studies, in vitro binding of the TtgT regulator to the *ttgGHI–ttgV* intergenic region has been demonstrated (Guazzaroni *et al.*, unpublished). Moreover, DNAse I footprinting assays have shown that TtgT and TtgV DNA binding sites within the same promoter region are similar which suggests that both regulators compete for the same operator in each efflux pump promoter (Terán *et al.*, unpublished; Guazzaroni *et al.*, unpublished).

No evidence of cross-regulation with the *ttgABC* operon has been detected. This is not surprising, as TtgR belongs to a different regulatory family (TetR), the inducers of the *ttgABC* efflux pump operon are different, and the promoter sequence shows low homology with that of *ttgDEF* or *ttgGHI*.

Although more experimental work needs to be done to clarify the importance of cross-regulation in solvent tolerance, these findings are the earliest evidence of cross-regulatory control of different RND efflux pumps involving their so-called "local" regulators. Studies of the affinity of these cross-acting regulators for different operators, or their ligand-binding properties, are needed to document the real effect of this cross-regulation on solvent efflux pump expression and on the response of *P. putida* DOT-T1E to organic solvents.

4. CONCLUDING REMARKS

Organic solvent tolerance in *Pseudomonas* is a very complex process. All bacterial solvent tolerance mechanisms should be tightly regulated to achieve the maximum level of tolerance. In a first step, cells present physical barriers to prevent the accumulation and toxic effects of organic solvents in the membrane. The activation of the Cti rapidly counteracts the fluidification effects of the solvent, although this mechanism is by itself not enough for the cell to survive and other resistance mechanisms (i.e., the efflux pumps) are also necessary.

Although considerable research has been done regarding the characterization of the Cti, several questions remain unanswered: how is a constitutive protein which seems to be permanently present and potentially active in the periplasm activated? And how is the molecular mechanism of the isomerization reaction? Besides, the Cti is present in organic solvent tolerant as well as in sensitive strains and although there will probably be no differences in the activation or functioning of the enzyme among the different strains, this comparative study has not been achieved yet.

Membrane lipids offer a promising tool as biomarkers for the analysis of microbial population changes during bioremediation of polluted sites. The level of *trans*-unsaturated fatty acids can be used as a marker for general stress during biodegradation processes[18, 22, 63, 76, 109, 112]. The system could also be used in the assessment of the general toxicity of organic compounds. This has already been described for aromatic carbonylic compounds[24, 30].

In the organic solvent-tolerant *P. putida* DOT-T1E strain, the pattern of expression and regulation of the three homologous solvent efflux systems *ttgABC, ttgDEF* and *ttgGHI* has been extensively explored[15, 86, 103], although intriguing new questions have arisen from these studies. The three efflux pumps function in an additive manner and the three operons are differentially expressed. These differences in gene expression correlate with the contribution of each system to the "intrinsic resistance" (*ttgABC* and *ttgGHI* are always expressed at a relatively high basal level) and "induced resistance" (*ttgDEF* and *ttgGHI* are solvent inducible), to organic solvents. This data raises the following question: is the expression of all three efflux pumps under coordinated regulation?

This hypothesis is supported by the fact that the induced levels of *ttgDEF* (in the presence of organic solvents) were higher in a single TtgGHI-deficient mutant (3-fold) and in a double mutant deficient in TtgABC and TtgGHI (7-fold) than in the wild-type strain (Terán *et al.*, unpublished). It seems that the cells are able to "sense" the lack of one efflux pump and compensate this deficiency by increasing the expression of other functional efflux pumps. Such coordinated expression has also been reported for the *mex*

multidrug system of *P. aeruginosa*: alterations in the expression level of one efflux system lead to compensatory changes in the expression levels of the others[58]. The question remains whether this overall compensation is the result of global regulation. Interestingly, the fact that in mutants deficient in the TtgR, TtgT or TtgV local repressors, the corresponding efflux pump operon (*ttgABC, ttgDEF* and *ttgGHI* respectively) is still induced by certain organic solvents, is a further indication of the existence of a higher level of regulation[86] (Terán *et al.*, unpublished).

Global stress and growth phase regulation have been reported to play a primary role in the control of *E. coli* efflux pump operons. The MarA and related stress regulators[3, 67], and more recently, the quorum-sensing regulator SdiA[79], have been shown to induce the *acrAB-tolC* solvent and multidrug efflux system. Although no *marA* homologs have been detected in the complete genome sequence of *P. putida* KT2440[68], this strain does contain a *sdiA* homolog. PCR amplification demonstrated that a very similar sequence is present within the *P. putida* DOT-T1E chromosomal DNA (Duque *et al.*, unpublished). This finding, together with the lines of evidence reviewed above, shows that we still have much to learn about the possible existence of a global regulation mechanism of solvent tolerance in *Pseudomonas*.

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18

TRANSCRIPTIONAL REGULATION OF THE TOLUENE CATABOLIC PATHWAYS

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1. TOLUENE DEGRADATION PATHWAYS IN PSEUDOMONADS

Although in the past century large amounts of aromatic hydrocarbons have been released as a consequence of industrial activity, most of these compounds in the environment are the product of the natural pyrolysis of organic material[28]. As a consequence, toluene, one of the simplest substituted aromatic compounds of natural origin, is widely distributed in natural environments. Therefore, because living organisms have been in contact with toluene and related compounds through evolutionary periods of time, it is not surprising that bacteria have developed the capability to degrade aromatic compounds such as toluene. In fact, aerobic bacteria able to use toluene as the sole source of carbon and energy are easily isolated from natural samples. Many of these organisms belong to the Pseudomonadaceae.

From a thermodynamic point of view, toluene is an extremely stable compound as a result of the high resonance energy of the benzene ring. A common strategy has been developed by microorganisms to weaken the aromatic ring

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prior to its cleavage: the introduction of two hydroxyl groups that destabilize the chemically stable resonant structure. However, bacteria have developed different molecular mechanisms to produce this di-hydroxylated compound. So far, five aerobic pathways have been described for the bacterial degradation of toluene, all of them leading to catechol or methylcatechols: The so-called TOL, TOD, TMO, TOM and TBU pathways. However, only three of them, the TOL, TOD and TMO pathways, have been found in *Pseudomonas* species. The enzymes that carry out the first reaction, that is, the direct insertion of one or two oxygen atoms in the toluene molecule, largely determine the pathway that is followed for degradation. The key steps involved in the pathways are briefly described below, and they are summarized in Figure 1.

The TOL pathway, coded by the archetypical plasmid pWW0[6] (Williams *et al.*, Chapter 6, Volume 1), is probably the best characterized pathway for toluene degradation from a biochemical and genetic point of view. This pathway is composed of two segments, an upper and a lower pathway. Through the upper pathway, the methyl group of toluene is sequentially oxidized to render benzoate. The first enzyme of this upper pathway is a toluene monooxygenase that oxidizes the methyl group of toluene to yield benzyl alcohol. Subsequent

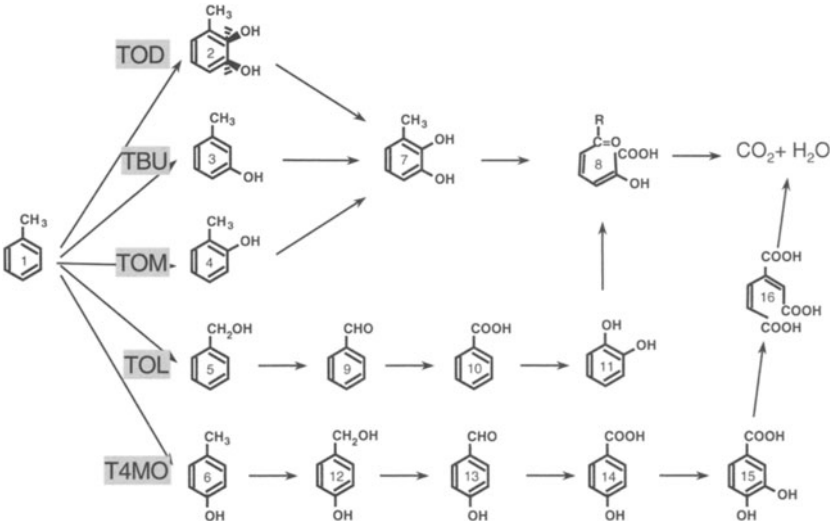

Figure 1. Pathways for the aerobic degradation of toluene. The intermediate compounds are 1, toluene; 2, *cis*-toluene dihydrodiol; 3, *m*-cresol; 4, *o*-cresol; 5, benzyl alcohol; 6, *p*-cresol; 7, 3-methylcatechol; 8, for R = H, 2-hydroxymuconic semialdehyde; for R = CH_3, 2-hydroxy-6-oxohepta-2,4-dienoato; 9, benzaldehyde; 10, benzoic acid; 11, catechol; 12, *p*-hydroxybenzyl alcohol; 13, *p*-hydroxybenzaldehyde; 14, *p*-hydroxybenzoic acid; 15, protocatechuic acid; 16, β-carboxy *cis*,*cis*-muconic acid. R, H or CH_3.

oxidation of the side chain is accomplished in two steps: first benzyl alcohol dehydrogenase renders benzaldehyde, which is further oxidized to benzoate by a benzaldehyde dehydrogenase. Benzoate then enters the lower pathway where the ring is dihydroxylated and cleaved in the *meta* position. For this reason, the lower pathway is also called the *meta* pathway.

A second degradation pathway found in *Pseudomonas* is the so-called TOD pathway, which was first described in *Pseudomonas putida* strain F1[46]. In this pathway, probably the best known in terms of the biochemistry involved, the first step is carried out by a three-component enzyme complex, the *to*luene 2,3-*d*ioxygenase (TOD), which renders *cis*-toluene dihydrodiol. This compound then undergoes dehydrogenation to yield 3-methylcatechol, the substrate for ring fission in the *meta* position (Figure 1). The product of ring cleavage is hydrolyzed and further degraded to enter the TCA cycle.

Finally, a third pathway was recently described in *Pseudomonas mendocina* KR1, where the first step in toluene metabolism is carried out by the *t*oluene-4-*mo*nooxygenase (TMO), which hydroxylates the aromatic ring in the *para* position to render *p*-cresol[130, 136]. In the subsequent steps, the methyl group is transformed by a methyl hydrolase, first rendering the alcohol and then the aldehyde derivative, which is finally oxidized to *p*-hydroxybenzoate by a dehydrogenase. The ring is further oxidized to 3,4-dihydroxybenzoate, which is the substrate for ring cleavage in the *ortho* position to enter the β-ketoadipate pathway.

As mentioned above, two additional pathways for toluene degradation with different initial reactions have been described in strains that were originally considered *Pseudomonas*: the so-called toluene-3-monooxygenase pathway (TBU), where toluene is first oxidized to *m*-cresol and then to 3-methylcatechol, and the toluene 2-monooxygenase pathway (TOM), where the first oxygen is inserted in the *ortho* position to render *o*-cresol, which is then oxidized to 3-methylcatechol (Figure 1). The original strains have been reclassified as *Ralstonia picketii* and *Burkholderia cepacia*, respectively, and an overview of the regulation of these pathways is presented at the end of this chapter. In addition, a slightly different pathway for the degradation of toluene was recently described in *P. stutzeri* OX1, a strain able to degrade *o*-xylene through an initial step that involves two successive monooxygenations of the aromatic ring carried out by the same enzyme, toluene-*o*-xylene monooxygenase (ToMO)[7, 9, 12]. This enzyme is interesting because of its broad substrate specificity and its relaxed regioselectivity, which make it able to hydroxylate more than one position of an aromatic substrate[12]. The organization of the genes involved in the degradation pathway is similar to that of the phenol degradation pathway of the *Pseudomonas* sp. strain CF600 *dmp* operon, and their master regulator is TouR, which belongs to the NtrC family of transcription regulators[4]. This regulator, homologous to XylR or DmpR, and

its activation mechanism are discussed by Shingler (Chapter 16, Volume 2), therefore they will not be considered in this chapter.

Although there are a number of different pathways to convert toluene to di-hydroxylated destabilized intermediates, the number of ring cleavage strategies identified to date is limited. Once the toluene ring is activated by one of the pathways mentioned above, two types of ring fission are possible: *ortho* or *meta* fissions. When the ring is opened between two hydroxy-substituted carbons (intradiol), a *cis,cis*-muconic acid is produced. This reaction is known as *ortho* cleavage[99]. On the other hand, when the ring is opened between one hydroxy-substituted carbon and a non-hydroxylated carbon (extradiol), the fission is known as *meta*-cleavage and the product formed is a semialdehyde[28]. As a general rule, alkyl-substituted catechols are degraded via the *meta*-cleavage pathway, whereas nonsubstituted or halo-substituted catechols are degraded via the *ortho*-cleavage pathway[51, 89].

A common feature to all toluene pathways from different bacteria is that the genes involved in the different reactions are organized as operons, which are either independent for the different segments of the pathways or transcribed as a single unit. In all cases, these pathways are under the control of regulatory mechanisms, which are ultimately modulated by toluene or intermediate substrates. This chapter will review the regulatory network that controls expression of the different pathway by depicting the situation in the model organism for each pathway. It is worth noting that our present understanding of the different pathways is lopsided, since some of them have been described only recently, while others have been under study for decades.

2. REGULATION OF THE TOL PATHWAY

In *P. putida* mt-2, the genetic information that determines growth on toluene, xylene and other alkyl derivatives is contained within the *xyl* operons of the TOL plasmid pWW0 (Williams *et al.*, Chapter 6, Volume 1). The *xyl* genes are organized in four transcriptional units: the upper and the *meta* operons and the *xylS* and *xylR* genes. The upper operon *xylUWCAMBN* codes for the enzymes necessary for the oxidation of toluene to benzoate; the *meta*-operon *xylXYZLTEGFJQKIH* encodes the enzymes for the oxidation of benzoate, the ensuing ring cleavage and the degradation to TCA cycle intermediates. The *xylS* and *xylR* genes, which are transcribed divergently, are located close to the *meta* operon 3′ end, and their products regulate the expression of the two catabolic operons. This pathway is no doubt the most extensively characterized regulatory system among the aromatic degradation pathways. This is not surprising if one considers the diversity and complexity of the regulatory mechanisms involved. Among these mechanisms are

two σ^{54}-dependent promoters, each with unique features, one regulator belonging to the NtrC and one to the AraC family of regulators, a σ^{32}/σ^{38}-dependent promoter, and several σ^{70}-dependent promoters, all of them under superimposed global control. As a whole, the TOL regulatory network can be considered a paradigm of integrated transcriptional regulation in prokaryotes.

2.1. Overview of the Regulatory Network

A scheme of the regulatory network that operates in the TOL pathway is presented in Figure 2. The model summarizes experimental evidence collected since the 1970s in different laboratories to explain the induction of enzymes of the pathway in the presence of toluene, benzoate or their derivatives. Two different regulatory circuits operate, depending on the nature of the aromatic compound present in the culture medium[108]. As summarized above, the toluene degradation pathway is divided into two segments: an upper pathway that transforms toluene into benzoate, and a *meta* pathway that utilizes benzoate as the first substrate and degrades it to TCA cycle intermediates. When cells are growing in the absence of any aromatic substrate, the *xylS* regulatory gene is expressed at low levels from the σ^{70}-dependent promoter Ps2, ensuring the presence of basal levels of XylS protein. This protein as such is not able

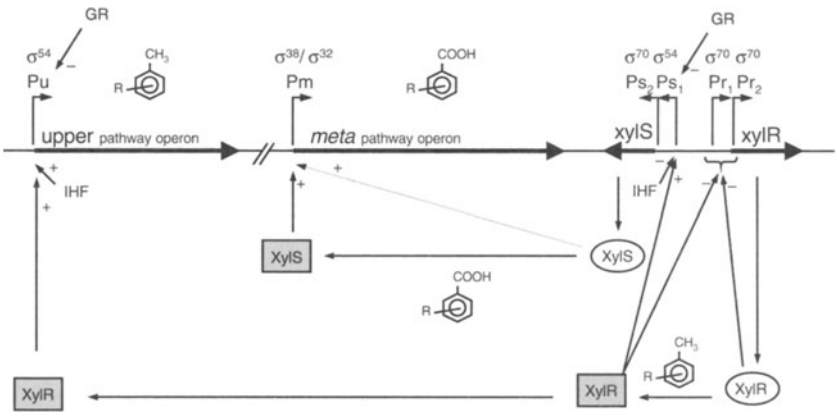

Figure 2. The TOL pathway regulatory network. Elliptical boxes indicate the inactive form of the regulatory proteins. Shaded square boxes indicate the active form of the regulatory proteins. Lines represent the connections between regulatory proteins and promoters, where (+) is activation of transcription and (−) is inhibition of transcription; GR, global regulation. The dotted line indicates transcription activation of overproduced XylS in the absence of effector. The sigma factor(s) involved in transcription initiation are indicated above each promoter. Aromatic substrates of the pathways that act as effectors of the regulatory proteins are indicated. The regulatory circuits are explained in the text.

to activate transcription. When a substrate of the *meta* pathway, for example, 3-methylbenzoate, is present in the growth medium, the XylS protein interacts with this compound and becomes active to promote transcription from the Pm promoter, which controls expression of the *meta* pathway. Expression from Pm requires RNA polymerase with either σ^{32} in the early exponential phase or σ^{38} thereafter. The XylR protein, which regulates its own transcription from two σ^{70}-dependent promoters, is synthesized in sufficient amounts under all growth conditions. When a substrate of the upper pathway, for example, toluene, is present in the culture medium, the binding of this effector to the protein triggers a series of molecular events that result in the activation of transcription from two σ^{54}-dependent promoters: Ps1 for the *xylS* gene, and Pu, which drives expression of the upper pathway. This latter activation requires the integration host factor (IHF). As a consequence of Ps1 activation, the XylS protein is overproduced, and even in the absence of a *meta* pathway effector, transcription from Pm occurs. The current knowledge of the molecular biology of each step on the regulatory pathway will be reviewed in detail below.

2.2. Regulation of the *meta* Pathway

Two main players are responsible for the expression of the *meta* pathway: the tandem Pm promoter/RNA polymerase and the tandem XylS/effector molecule. Extensive genetic data have been obtained to determine the architecture of Pm promoter and the mechanisms through which the regulator becomes active to promote transcription from Pm. As stated above, basal levels of the XylS protein are guaranteed under any growth conditions by the activity of the constitutive σ^{70}-dependent promoter Ps2. In the presence of 3-methylbenzoate, the XylS protein becomes active to promote transcription from Pm.

2.2.1. The XylS Regulator

XylS belongs to the AraC/XylS family of transcriptional regulators, which includes at least 284 different proteins[36b, 43, 110, 125] that participate in diverse cellular functions such as carbon metabolism, pathogenesis or response to alkylating agents. In general, proteins of this family consist of two domains: a 100 amino acid conserved domain involved in DNA binding (the C-terminal domain in most of the proteins of the family, including XylS), and a nonconserved domain (the N-terminal domain in XylS) involved in effector binding and dimerization.

Interactions between XylS and its effector have been studied by analyzing the ability of the protein to activate transcription in the presence of a wide range of substituted benzoates, as well as by selecting XylS mutants with altered effector specificity[91, 109, 111, 138]. Recognition of ring substituents strongly depends on the position and nature of the chemical change, with *meta*

being the most permissive position in the aromatic ring[111]. These analyses, reviewed in details elsewhere[108], led to the identification in the XylS N-terminus of key residues clustered in two noncontiguous segments involved in effector recognition. Recent studies further pinpointed residues Asp137 and His153 as crucial for interactions with the effector molecule[115]. In addition to influencing effector specificity, these two residues were shown to contact specific residues in the RNA polymerase α subunit carboxy terminal domain (α-CTD)[115]. Interaction with α-CTD has also been observed in other members of the family[14, 77, 82]. Within the family, some regulators such as MarA are present in solution as monomers, whereas most of the members of the family are found as dimers in solution[78, 105, 113, 122]. As in AraC or UreR, XylS exhibits a series of conserved leucines in the sequence that connects the N- and C-terminal domains of the protein (the linker domain). Both in vivo and in vitro assays have shown that this domain is responsible for protein dimerization, which requires the presence of an effector molecule, and that Leu193 and Leu194 play a crucial role in this process[116].

The DNA binding domain of XylS consists of seven α-helix units which fold to assemble two helix-turn-helix (HTH) motifs that interact with two neighboring major grooves on one face of the target DNA. Involvement of the XylS C-terminal domain in DNA binding was first predicted after the finding of mutations in this domain that rendered mutant regulators able to promote high transcription levels in the absence of effectors[109, 138]. Mutation analysis of the conserved positions of the HTH motifs of XylS showed that the most conserved positions in the family reflect structural requirements of the domain[84]. Deletion of the 209 N-terminal residues of XylS rendered a C-terminal domain-protein able to bind Pm promoter and, when overproduced, able to activate transcription in vivo to levels similar to those in the wild-type protein. However, activity was clearly reduced when the C-terminal fragment was synthesized at physiological levels. As expected, the truncated protein was not responsive to effector-mediated control[68].

In *P. putida* KT2440, an mt-2 derivative that lacks the TOL plasmid and therefore the *xylS* gene, some activation of the Pm promoter is observed when benzoate is added. This activity is the consequence of cross-talk regulation mediated by BenR, a regulator homologous to XylS coded by *P. putida* chromosome and responsible for the regulation of an *ortho* pathway for benzoate degradation present in this strain[27, 63, 71].

2.2.2. The Pm Promoter

XylS-mediated transcription activation from Pm requires a DNA fragment extending to position −70 upstream from the transcription start site. The DNA in this region exhibits a 40° bend centered between positions −41 and −46[49]. The XylS binding site in the Pm promoter was first defined through

site-directed mutagenesis[42, 50, 69, 70] and further confirmed by in vitro and in vivo footprint assays[50, 67, 92]. The XylS binding site in Pm consists of two directed repeats (5′-TGCAN$_6$GGNTA-3′) spanning positions −34 to −68 upstream from the transcription start site, and overlapping the RNA polymerase binding site by 1 bp[49, 50]. This overlap with the RNA polymerase binding site is also observed in several other members of the family[5, 15, 32].

In vivo transcription from Pm is mediated by two different RNA polymerases, depending on the growth phase. In the early exponential phase, RNA polymerase with σ^{32} is necessary for transcription, whereas in late exponential growth phase and in stationary phases, sigma factor σ^{38} is required[85, 87, 92]. Despite the alternation in RNA polymerase, the same transcription start site is detected along the growth curve, suggesting that the same promoter is used by both forms of RNA polymerase. Transcription of σ^{32}-dependent promoters requires the stabilization of this sigma factor, which takes place through a series of events known as heat-shock response[137]. In the TOL pathway regulatory network, aromatic effectors are required not only because of their direct role in XylS activation to promote transcription, but also to trigger the heat-shock response and provide the appropriate RNA polymerase for transcription in the exponential phase[87]. The direct involvement of the two sigma subunits in Pm transcription was further supported by the finding that a mutant Pm promoter with an altered XylS binding site, combined with the mutant regulator XylSGly44Ser, was able to overcome the requirement of σ^{38} for transcription in the stationary phase[85]. Moreover, the mutants XylSAsp137Glu and XylSHis153Gln were able to stimulate transcription from Pm in the absence of σ^{38} (Ref. 115). Recent experimental evidence indicates that specific bases in the proximal region of Pm promoter may play a key role in discriminating between the sigma factors involved in transcription at any given moment (Domínguez-Cuevas *et al.*, unpublished). However, an additional role of sigma competition for core binding in RNA polymerase switching in Pm cannot be ruled out at the moment.

Expression of the *meta* pathway can be envisaged as the consequence of two sets of events. On one hand, the XylS protein must enter into an active state through interaction with the effector molecule, which would probably assist dimerization or increase XylS affinity for its target DNA. On the other hand, the RNA polymerase with a specific sigma factor (depending on the growth phase) must interact with Pm promoter and await activation by the XylS regulator. Although experimental evidence suggests that XylS binds its target sequence as a dimer, and that the effector is required for dimerization, it is not yet clear whether this process takes place in solution or whether it requires that the protein previously binds to DNA. In vitro gel shift assays suggest, however, that XylS is capable of promoter binding in the absence of effector (Domínguez-Cuevas *et al.*, unpublished). Thus, the effector may interact with

XylS bound at its target sequence, changing its conformation to an active state proficient in transcription activation. The interaction of XylS with α-CTD may in turn influence interactions between XylS and the sigma subunit of the RNA polymerase, and hence modify the requirement for a specific sigma factor. It is worth noting that interactions between the alpha and sigma subunits of the RNA polymerase were recently described[114]. As mentioned above, the effector molecule plays an additional role in activation of Pm, influencing the level of the different sigma factors. The alternative sigma factors show a lower affinity than σ^{70} for RNA polymerase core[83]. This underscores the importance of the consequences of sigma competition for RNA polymerase core binding, which plays a crucial role in promoter selectivity in vivo[64a, 83]. Thus, after an effector is added, the cellular concentration of σ^{32} is expected to increase, thus increasing in turn the fraction of RNA polymerase bound to this sigma factor able to transcribe from Pm. Entry into the stationary phase increases the amount of σ^{38} in the cell, allowing this sigma factor to compete advantageously for the RNA polymerase core and transcribe from Pm.

2.3. Regulation of the Upper Pathway

In the presence of toluene or a substrate of the upper pathway, *P. putida* ensures the coordinated expression of the two catabolic operons, so that the aromatic compound is totally degraded to TCA intermediates. The key regulator in this process is XylR, which is responsible for the coordinated expression from the σ^{54}-dependent promoters Pu and Ps1. Pu drives transcription of the upper pathway and Ps1 increases the synthesis of XylS, responsible for *meta* pathway expression (Figure 2).

2.3.1. The XylR Regulator

XylR protein belongs to the class of regulators known as the NtrC family of enhancer-binding proteins (EBP)[62, 75, 95]. It presents the four domains distinctive of this family. An N-terminal A-domain is responsible for signal reception, that is, interaction with the effector molecule (see below). The A-domain is linked to the central domain, called domain C by the short B-domain (Q-linker), structured as a coiled-coil and probably involved in oligomerization[44, 97]. The C-domain is involved in ATP binding and hydrolysis, and plays a major role in the isomerization of the σ^{54}-dependent promoters from close to open complexes. A C-terminal D-domain contains the HTH motif for DNA binding.

XylR is activated by aromatic compounds with a wide variety of substitutions such as alkyl groups of different length or oxidized intermediates of the toluene methyl group, such as benzyl alcohol, benzaldehyde and derivatives[2, 30]. Early evidence indicated that the A-domain was the signal receptor from the

environment and the direct sensor of the aromatic molecule. This was surmised from the ability of the protein to activate transcription from the Pu promoter in the presence of a wide range of toluene derivatives, and by experiments with XylR mutants with altered effector specificity[2, 30, 31, 45]. These data, obtained in the heterologous host *E. coli*, led to the conclusion that XylR was directly activated via interaction with the effector. XylR is closely related to the DmpR regulator for phenol degradation in *Pseudomonas* sp. CF600, which recognizes phenol and derivatives, but not toluene, as an effector (Shingler, Chapter 16, Volume 2). Further evidence for the direct interaction of the A-domain of these proteins with the effector molecule came from the construction of a chimeric protein in which the receptor domain of DmpR was replaced by the corresponding domain of XylR, resulting in a hybrid regulator that responded to toluene for activation of the P*o* promoter of the phenol degradation pathway[121].

The A-domain operates as an intramolecular repressor of the central activating domain of the protein[38, 102]. In fact, a XylR derivative in which the A domain has been deleted is able to activate Pu in the absence of an aromatic effector. These findings, together with a thorough analysis of DmpR wild-type and variant protein interaction with different aromatic compounds, made it possible to develop an interesting model to explain this intramolecular repression, according to which the Q-linker coordinates transmission of the effector signal to the central domain[44, 94, 102, 120, 131] (Shingler, see Chapter 16, Volume 2). DNA shuffling assays to create hybrid A-domains between DmpR and XylR confirmed that the residues 110–186 of both proteins were responsible for the effector profile of these regulators[121].

The truncated derivative of XylR depleted of the A-domain and therefore unable to respond to effector-dependent modulation showed intrinsic ATP binding and hydrolysis activity, located in the central activation domain (C-domain). This activity was strongly stimulated by the presence of a DNA fragment containing the native XylR binding site in Pu (upstream activator sequences [UAS], see below)[103]. Furthermore, binding of ATP to this truncated protein alone was able to induce conformational changes in the protein. Initially, a cyclic model to explain XylR activation of Pu was proposed by Pérez-Martín and de Lorenzo[104], according to which ATP binding to the XylR central domain led to multimerization of the regulator bound to its UAS in Pu, followed by ATP hydrolysis. This in turn triggered σ^{54}-dependent transcription initiation in Pu, allowing the system to return to its initial disassembled state[104]. Recently, Shingler and coworkers studied the analogous regulator DmpR, and suggested an alternative mechanism to explain effector-dependent activation of σ^{54}-dependent promoters. According to their model, DmpR dimers are activated after binding of the effector molecule to the A-domain, followed by a conformational change that allows ATP binding to the central

domain and oligomerization to a hexameric conformation, probably required to promote transcription initiation. Finally, ATP hydrolysis leads to dissociation of the hexameric structure and dissociation of the effector.

2.3.2. The Pu Promoter

Pu promoter belongs to the class of promoters dependent on the alternative sigma factor σ^{54}, and shows the architectural organization typical of the promoters of this class. A $-12/-24$ sequence is responsible for the recognition of σ^{54}-RNAP[34, 60], and UAS ensure XylR binding between positions -120 and -175[1, 29]. An IHF binding site between these two elements (positions -52 to -79)[29] is needed to bring the regulator into contact with the RNA polymerase by looping out the corresponding binding sequences. Direct evidence that IHF causes Pu to form an open bend was obtained by atomic force microscopy, where an angle of 123° was measured between the UAS and the RNA polymerase binding site[118]. However, Bertoni *et al.*[10] recently found that a second upstream element, reminiscent of the so-called α-CTD-binding UP elements of σ^{70}-dependent promoters, was important in Pu recognition by σ^{54}-RNAP, and that IHF-binding played an additional role in Pu. This role consisted of σ^{54}-RNAP recruitment to its binding site, determined by the correct positioning of the UP-like element with respect to the $-12/-24$ binding site after IHF-dependent DNA bending[10]. That IHF-mediated σ^{54}-RNAP recruitment provided enhancement of Pu transcription was further supported by the fact that the effect was reproduced in vitro with the XylR regulator acting from solution, that is, in the absence of UAS[18]. These authors have shown that RNA polymerase binding is an important rate-limiting step in Pu activation, which could become crucial when limiting concentration of enzyme are present[18].

2.4. Expression of the Regulatory Genes

The level of the XylR and XylS proteins is finely modulated in vivo. This fine regulation takes place in the 300-bp intergenic region between the *xylR* and the *xylS* genes, which contains four promoters: the two σ^{70}-dependent tandem promoters of *xylR*, Pr1 and Pr2, divergent from the two that drive transcription of *xylS*, the σ^{54}-dependent promoter Ps1 and the σ^{70}-dependent promoter Ps2. The binding sites for the different regulatory proteins in this short region overlap totally or partially. Thus, XylR UASs in Ps1 partly cover the two RNA polymerase binding sites of Pr1 and Pr2. In addition, two sequences with different affinity for IHF are found which overlap the Ps1 $-12/-24$ RNA polymerase binding site and one of the UAS[54, 55, 57]. As a consequence, the levels of expression normally observed in the wild-type strain for each promoter are below maximum values, indicating the involvement of repressive element(s) in the maintenance of appropriate levels of expression.

In fact, as expected from the promoter architecture described above, XylR strongly represses its own synthesis[2, 11, 13, 48, 61, 88]. Activation of the Ps1 promoter and auto-regulation of XylR expression seem to be the consequence of the binding of XylR to the UASs for Ps1 that overlap the Pr1 and Pr2 promoters. This is in agreement with the finding that XylR is consistently bound to target sequences[2]. Thus, *xylR* promoters may be subjected to two levels of repression depending on the mechanism of XylR activation discussed above: an ATP-independent level resulting from non-cooperative interaction of nonactivated XylR with the Ps1 UAS[13], and an ATP-dependent repression level resulting from the cooperative oligomerization of activated XylR at the UAS in Ps1[11]. As soon as the protein is activated and the UAS is strongly bound by the regulator, XylR expression is minimized, thus limiting the period of time during which the Ps1 and Pu promoters of the TOL plasmid are in an activated state[11].

The role of IHF in Ps1 expression deserves special attention. Analysis of Ps1 activity in isogenic IHF-plus and minus backgrounds showed that in the presence of toluene, the highest levels of expression were found in the absence of IHF[88]. This may reflect a better access of either XylR to its binding site or of σ^{54}-RNA polymerase to the $-12/-24$ region of Ps1, or both. On the other hand, it may be the consequence of structural hindrance, as the DNA bends induced by the IHF protein bound to the two sites may give rise to a highly ordered structure that restricts the access of regulatory proteins to the corresponding promoters. The high level of expression from Ps1 in the IHF-minus background in the presence of effectors contrasts with the diminished expression from the TOL plasmid Pu promoter for the upper pathway in an IHF-deficient background. The most noticeable difference between the two promoters is the position of the IHF binding site, which in Pu lies between the UASs and the $-12/-24$ box. In addition to affecting Ps1 expression, the close proximity of the regulatory sequences in the intergenic region results in a high expression level from Ps2 in the absence of σ^{54}, that is, when RNA polymerase is unable to bind to the Ps1 promoter[88].

In general, the physiological consequence of this organization is that in the absence of any effector in the culture medium, Ps2, Pr1 and Pr2 promoters are slightly repressed. In the presence of toluene, activation of Ps1 causes a stronger repression of both *xylR* promoters. As a result, the level of XylR is maintained at approximately 30 monomers per cell[41], which are apparently sufficient to promote high expression of both *xylS* and the upper pathway. Under these conditions, the XylS protein is overproduced, which allows induction of expression from the Pm promoter even in the absence of *meta* pathway substrates. Therefore, in the presence of toluene or a substituted derivative, both the upper and the *meta* pathways are coordinately expressed to optimize total degradation of the aromatic.

2.5. Integration in the Bacterial Metabolism

The expression of the TOL pathways is tightly regulated according to the carbon sources available for growth. This has been observed repeatedly since the first description of the pathway, and experimental evidence of regulation have been obtained through a variety of methods and growth conditions[35, 36a, 56, 58, 86, 133]. The regulation is exerted mainly at the level of the two σ^{54}-dependent promoters Pu and Ps1, and was first observed as a delay in the induction of expression from these promoters when cells were induced in a rich complex medium[58, 86]. Since both promoters were silent in this medium during rapid exponential growth, and expression appeared only at the end of the exponential phase, this behavior was named exponential silencing[21]. However, it is worth noting that exponential silencing is only observed in rich medium; in defined minimal media with succinate (for example) as a carbon source, expression of both Ps1 and Pu is observed immediately after induction[19, 58, 86]. Growth rate as a determinant of Pu and Ps1 expression was ruled out through a series of continuous culture experiments that compared different growth rates controlled by limiting different substrates. The results led to the conclusion that repressive conditions correlated with a high energy status of the cells[35, 36a]. In fact, when the growth-limiting factor was an anabolic substrate (i.e., P, S or N), a situation predicted to generate a high energy level[52], expression from Pu was totally repressed. In contrast, when carbon (a catabolic substrate) was the growth-limiting factor, thus creating conditions of low energy level in the cells, promoters were induced at maximal levels[36a]. In other words, in all conditions tested where excess carbon was available, the system was repressed. However, when the catabolic substrate oxygen was the growth-limiting substrate, a situation where carbon was also present in excess, a certain degree of derepression was observed although activity never reached maximum values.

In batch cultures in minimal medium amended with casamino acids, carbon sources such as glucose, gluconate or α-ketoglutarate inhibited expression from Pu. In general, this phenomenon reflects the preferential and sequential use of the different carbon sources present in a mixture; hence it could be considered a typical case of catabolite repression. However, catabolite repression in *Pseudomonas* seems to be exerted through mechanisms that differ greatly from the classical CRP-dependent phenomenon observed in *Escherichia coli*. Similar physiological regulations of catabolic operons has been observed in other pathways in *Pseudomonas*, and are reviewed in detail elsewhere (Rojo and Dinamarca, Chapter 13, Volume 2; Shingler, Chapter 16, Volume 2).

Although the molecular basis for the observed repression of Pu and Ps1 expression has been under study since the early 1990s, no clear conclusion about the processes involved can be drawn yet. Several alternatives have been

envisaged and the current picture is compatible with the partial involvement of different systems in the global regulation response. Originally, the phenomenon known as exponential silencing was shown to be due neither to a late activation of XyIR by the aromatic effector nor to changes in the intracellular levels of IHF during growth[21]. However, recent findings obtained with in vivo UV laser footprint technology have shown that IHF occupancy of its target site in Pu increases upon entry into the stationary phase, in parallel with an increase in IHF concentration in the cell. Therefore, this could explain the increase in Pu activity with growth phase in batch cultures. Nevertheless, these results do not preclude the integration of physiological repressive signals through additional mechanisms[127].

In *P. putida* CF600, (p)ppGpp plays a key role in catabolite repression of DmpR-activated P*o* expression (Rojo and Dinamarca, Chapter 13, Volume 2; Shingler, Chapter 16, Volume 2). Due to the substantial similarity between P*o* and Pu physiology and activation mechanisms, the possible involvement of the stringent response mediated by the alarmone (p)ppGpp in control of the Pu promoter was examined in vivo and in vitro. Although Pu activity was indeed stimulated by ppGpp, the effect observed was insufficient to explain Pu inhibition during exponential growth. The moderate effect of ppGpp on Pu seems to be rather the result of direct stimulation of the transcription initiation complex by the alarmone. Thus, differences between Pu and P*o* regulation may reflect differences in the architecture of the two promoters, which make them sensitive to different types of metabolic signals[19] (Rojo and Dinamarca, Chapter 13, Volume 2).

The σ^{54} factor of RNA polymerase has also been considered a possible target of global regulation. Overproduction of the σ^{54} factor allowed Pu to partially overcome exponential silencing, although not carbon source-dependent repression[21]. Since σ^{54} protein levels remained approximately constant during growth under physiological conditions[65], exponential silencing of Pu may be caused ultimately by changes in the activity of the sigma factor itself. The ATP-dependent physiological protease FtsH, a member of the so-called AAA family of ATPases responsible for the stability of various transcription factors such as σ^{32} (Ref. 126), has been shown to play a key role in Pu expression. FtsH is required for XylR-mediated *Pu* transcription in a process that is not related to XylR or IHF, but which is rather exerted through a mechanism that involves the loss of σ^{54} activity. In fact, overproduction of σ^{54} restored about 60% of *Pu* activity in the absence of FtsH[17]. Furthermore, the overproduction of FtsH partially relieved exponential silencing of Pu expression. In this connection, the target of FtsH activity was seen as an additional factor that down-regulated σ^{54} post-translationally, or that hindered the contacts of σ^{54}-RNAP with the promoter or with the activator. Interestingly, *E. coli* FtsH levels are controlled in response to physiological signals and its proteolytic function is stimulated by the proton-motive force[3].

Finally, exponential silencing and carbon source-dependent repression can be distinguished at a genetic level. The *rpoN* gene, which codes for the σ^{54} sigma factor required for Pu activity, is the first gene of an operon found in gram-negative bacteria that includes four additional ORFs. Two genes of this cluster, *ptsN* and *ptsO*, code for two proteins, IIA^{Ntr} and NPr, respectively, that show similarity to phosphotransferases belonging to the phosphoenol pyruvate:sugar phosphotransferase system (PTS) family. To understand the putative role of the ORFs in this cluster in the global control of Pu, knock-out mutants were generated and analyzed. A mutant in the *ptsN* gene (which encodes IIA^{Ntr}) relieves C source inhibition, but not the exponential silencing of Pu[20]. The *ptsO* gene together with *ptsN* operates in Pu regulation, where phosphorylation of the *ptsO*-encoded protein NPr is necessary for the normal response of Pu to glucose[22, 24]. NPr probably modulates IIA^{Ntr} activity, promoting its dephosphorylation. This increases the concentration of unphosphorylated IIA^{Ntr}, and as a result inhibition of Pu disappears. Interestingly, a site-directed *ptsN* mutant in the conserved phospho-acceptor His-68 residue made Pu unresponsive to the presence or absence of glucose, thus supporting the notion that phosphorylation of IIA^{Ntr} mediates the C source inhibition of the promoter[23].

The observations reported above suggest that the global regulation of TOL pathway expression responds to several complex mechanisms that act at the level of σ^{54}-dependent promoters. An interesting comparison of the putative mechanisms that underlie this regulation in the parallel σ^{54}-dependent systems Pu/XylR and Po/DmpR was carried out recently by using combinations of the specific regulatory elements for both systems and making use of their ability to cross-regulate each other[123]. A corollary of this analysis was that the specific regulator of each system (i.e., XylR or DmpR, respectively) determined the host regulatory mechanism used to respond to physiological signals. An interesting discussion on the processes involved in each case and the differences between the systems can be found in Chapter 16 of this volume.

3. REGULATION OF THE TOD PATHWAY

Pseudomonas putida strains F1 and DOT-T1E use benzene, toluene and ethyl benzene as the sole carbon source[47, 107]. The enzymatic pathway responsible for converting these aromatic hydrocarbons to TCA cycle intermediates has been well characterized biochemically and genetically[40, 47]. The catabolic genes for the complete conversion of toluene to TCA cycle intermediates are clustered in a single unit, the *tod* operon, as *todXFC1C2BADEGIH*[79, 90, 93, 129, 140, 141] and these genes are coordinately induced by toluene[39, 129]. A putative regulatory gene, *todR*, with sequence homology to the DNA-binding domain of

transcriptional activators of the LysR family, is found upstream from *tod*X. However, the *todR* sequence is truncated, and as expected, plays a negligible role in *tod* operon regulation[129].

3.1. A Two-Component Signal Transduction System Controls *tod* Operon Expression

Wang and coworkers[129] and Mosqueda and coworkers[93] identified a single promoter upstream from the *tod*X gene, whose −10 and −35 regions showed homology with *P. putida* σ^{70}-dependent promoters (Domínguez-Cuevas and Marqués, Chapter 11, Volume 2). Expression of the *tod* catabolic operon is regulated by *todST* gene products, which are located as a separate transcriptional unit downstream of *todH*, the last gene of the operon. Translational coupling between *todS* and *todT* ensures the balanced transcription of both genes[79]. TodS and TodT proteins belong to the family of two-component signal transduction systems. The regulation mechanism of two-component control systems is based on a histidine-aspartate phosphorelay circuit working between the two components. One of them is a sensor that autophosphorylates in response to an external signal, and the other one is the so-called response regulator, which receives the phosphate from the former and activates transcription. TodS, the 108-kDa sensor protein, is a member of the hybrid class of histidine kinases and possesses multiple protein domains (Figure 3). (a) The N terminus of TodS contains a motif characteristic of the *b*asic region leucine *zip*per (bZIP), which, although common among transcriptional factors in eukaryotes, is rarely found in prokaryotes[59], (Ventre et al., Chapter 9 in this volume). The bZIP motif consists of a region with several basic residues which probably contact DNA, and an adjacent region containing a heptad repeat of leucine, the leucine zipper. Indirect evidence suggests that in TodS, the leucine zipper mediates dimerization, which is required for DNA binding[79]. (b) A duplicated histidine kinase motif, each element of which is characterized by five short sequence blocks that are highly conserved (Figure 3). These elements flank (c) a receiver domain of the response regulator located at the center of the protein adjacent to one set of a PAS domain, known as a signal sensor and found in various redox, light

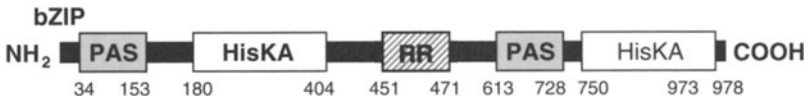

Figure 3. Schematic representation of *P. putida* F1 TodS protein. The numbers of amino acids in each domain are deduced from the web-based SMART program provided by http://smart.embl-heidelberg.de. bZIP, basic leucine zipper motif; PAS, PAS domain; HisKA, histidine kinase domain; RR, receiver domain of the response regulator. Reproduced with permission from ref. [25].

and hydrocarbon sensor proteins[124, 139] (Figure 3). The *todT* gene encodes a 206-residue protein with an estimated mass of 23 kDa. Analysis of the amino acid sequence of TodT shows significant sequence similarity with response regulators of two-component signal transduction systems[53, 112]. TodT consists of an N-terminal receiver domain to accept the phosphoryl group from TodS, and a HTH DNA-binding domain. The TodT protein was shown to specifically bind to the *todX* promoter region at a 6-bp inverted repeat located 105 bp upstream from the transcription start site, and known as the *tod* box[79].

This two-component signal transduction system controls the positive regulation of the *tod* operon[79]. TodS is predicted to function as a hybrid kinase that uses ATP to autophosphorylate at a specific histidine residue in response to toluene (H-190 and H-760 are the predicted sites in TodS). TodT as a response regulator probably receives the phosphate at a conserved aspartate (Asp-56) and then mediates transcription activation at the *todX* promoter. Although biochemical evidence supporting this model is consistent[79], in vivo studies that confirm the role of each member of the tandem are scarce. Furthermore, the physiological behavior of this system under different growth conditions and the precise mechanism through which the presence of toluene triggers changes in *tod* gene expression in vivo are not completely understood. For example, in a heterologous system such as *E. coli*, TodT, probably in its phosphorylated form, is able to activate the *tod* operon in the absence of TodS protein[79]. This is explained in terms of metabolic cross-talk, probably carried out by unrelated *E. coli* sensor protein(s). Similar results have been observed with *P. putida* DOT-T1E TodT expressed in *E. coli* (Lacal *et al.*, unpublished). On the other hand, the role of TodS as a sensor for directly detecting inducers has not been clearly demonstrated, nor has the role of these proteins in self-regulation been clarified. Expression from the *todX* promoter occurred in response to toluene, ethyl benzene, styrene, xylenes and other aromatic hydrocarbons, although the greatest level of expression was obtained with toluene. Expression from the *todS* gene was constitutive regardless of which aromatic was tested[93]. Some amino acid residues in TodS have been identified as responsible for the recognition of new effectors that are not recognized by the wild-type protein. These amino acids are found in the regions corresponding to the receiver domain and the second PAS domain in the TodS protein[25]. These results point to a direct interaction of TodS with the effector molecule. However, the specific functions of these positions remain to be elucidated.

Interestingly, the presence of a receiver domain and two PAS domains in TodS implies that the sensor can recognize different environmental or intracellular signals. It has been suggested that TodS is a dual sensor that responds primarily to toluene, but also able to sense either oxidative stress or oxygen concentration in the environment[79]. In this regard *todX*, the first gene in the TodS-regulated *tod* operon (which codes for an outer membrane protein) may

play a role in the signal transduction process triggered by the presence of toluene. It is interesting to note that both TodS and TodT proteins are required for chemotaxis to aromatic hydrocarbons in *P. putida* F1[101]. This observation indicates that both catabolism and chemotaxis are coordinately regulated at the transcriptional level. The functions of each domain in the aromatic hydrocarbon sensor have not been identified experimentally, with the exception of that of the leucine zipper dimerization motif from TodS in *P. putida* F1[79].

3.2. Two-Component Systems in Aromatics Degradation Pathways

The involvement of a two-component signal transduction system as a transcriptional regulatory unit in the *tod* pathway is interesting, because many other degradation pathways of aromatics use single component transcriptional regulators[33]. However, since the mid-1990s, an increasing number of pathways have been shown to be regulated by two-component systems in prokaryotes. This is also the case for the aromatic degradation pathways in bacteria. In addition to toluene degradation through the *tod* pathway, similar two-component signal transduction systems regulate styrene degradation in several *Pseudomonas* strains[96, 100, 117, 128], biphenyl degradation in *Rhodococcus* sp. strain M5[76], and anaerobic toluene degradation in the denitrifying strains *Thauera* T1 and *T. aromatica* strain K172[26, 81]. However, the sensor proteins found in anaerobic toluene degradation are shorter than TodS and contain only one histidine kinase domain.

4. REGULATION OF THE TOLUENE-4-MONOOXYGENASE PATHWAY

Genes involved in toluene degradation in *P. mendocina* KR1 are organized in an unusual manner: the five peptides that make up the multicomponent enzyme toluene-4-monooxygenase, which carries out the primary oxidation of toluene to *p*-cresol (Figure 1), are coded by the *tmoXABCDEF* gene cluster[135, 136], where *tmoX* is homologous to the outer membrane protein that codes *todX* from *P. putida* F1[106]. Complete toluene oxidation to TCA cycle intermediates requires another operon, *pcuCAXB* for *p*-cresol oxidation to *p*-hydroxybenzoate, and the *pobA* gene, which presents two alleles, *pobA1* and *pobA2*, for the further transformation of this compound into protocatechuate. The *pcuCAXB* and *pobA* genes are not clustered with the *tmo* genes on the *P. mendocina* chromosome. These operons and the *pobA* genes are also independently regulated[134]. The regulatory elements involved in the toluene degradation pathway in *P. mendocina* KR1 are summarized in Figure 4.

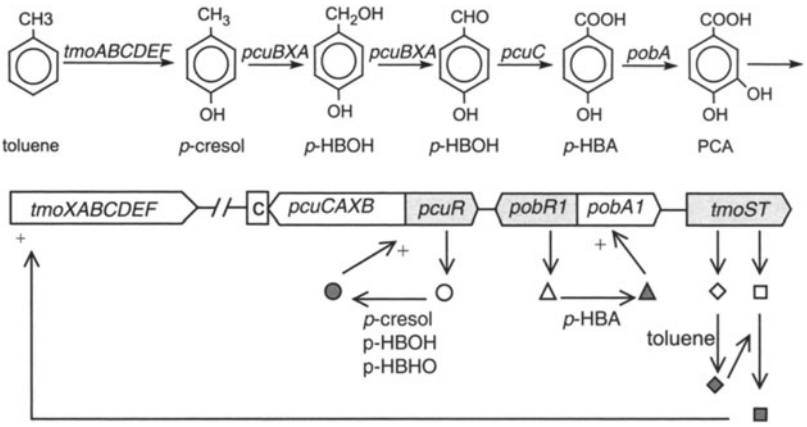

Figure 4. Regulation of toluene degradation in *P. mendocina* KR1. The upper part of the figure summarizes the oxidation of toluene to protocatechuate and the genes needed for each reaction. The lower part shows a scheme of the cluster of *tmo*/*pcu*/*pob* genes in this strain. *tmoABCDEF*, toluene-4-monooxygenase genes; *c*, putative cytochrome *c* gene; *pcuRCAXB*, *p*-cresol utilization genes; *pobR1* and *pobA1*, regulator and *p*-hydroxybenzoate hydroxylase, respectively; *tmoST*, two-component signal transduction system; *p*-HBOH, *p*-hydroxybenzyl alcohol; *p*-HBHO, *p*-hydroxybenzyl aldehyde; *p*-HBA, *p*-hydroxybenzoate; PCA, protocatechuate. Reproduced with permission from ref. [106].

4.1. Regulation of *tmo* Operon Expression

The transcription initiation point from the *tmo* operon has been mapped and the sequence upstream has revealed strong identity with the promoter of the *tod* operon of *P. putida* F1, including an inverted repeat located at position −100 and an almost identical *P. putida* F1 *tod* box. This suggested the involvement of a regulatory system similar to TodS-TodT for the transcriptional control of the *P. mendocina* toluene degradation pathway. In fact, a novel two-component signal transduction system was recently described in *P. mendocina* KR1[106]. Transcription from the P_{tmoX} promoter, which directs the expression of the *tmoXABCDEF* gene operon, is induced in the presence of toluene or *p*-cresol by a two-component system made up of TmoS and TmoT, which are 83% and 85% identical, respectively, to the TodS and TodT proteins described above. Furthermore, transcription from P_{tmoX} and P_{todX} can be mediated by TmoS-TmoT or TodS-TodT in the presence of toluene, revealing cross-regulation between these two catabolic pathways. In the absence of the two-component regulatory system, no expression of the *tmo* operon is observed, thus confirming the role of *tmoST* as primary regulators. Surprisingly, in *P. mendocina* the *tmoST* genes are not located close to their target operon *tmoXABCDE*, as is the case in *P. putida* F1, but are found

approximately 700 bp downstream from the *pobA1* gene for *p*-hydroxybenzoate hydroxylase, in a different region in the chromosome (Figure 4). It is interesting to note that truncated sequences showing homology to transposases are found downstream from *tmoST* and upstream from the *tmo* operon (Ramos-González, unpublished). In fact, early mobilization experiments found good linkage between the *pcu* and *pob* operons together with their corresponding regulatory genes, but no linkage between this cluster and the *tmoXABCDE* operon[134]. Sequencing of the *tmoST* surrounding region recently confirmed this distribution[106].

4.2. Regulation of the Lower Segments of the Toluene Degradation Pathway

In *P. mendocina* KR1, the further degradation of *p*-cresol to TCA cycle intermediates requires the catabolic operons *pcuCAXB*, *pobA* and the gene necessary for ring opening and subsequent degradation. Current knowledge of the regulation of these operons is limited and most studies have been carried out to obtain recombinant strains able to overproduce *p*-hydroxybenzoate[8].

The *pcuCAXB* operon is required to transform the *p*-cresol produced from toluene by toluene monooxygenase into *p*-hydroxybenzoate. The *pcuCAXB* operon is regulated by the divergently transcribed *pcuR* gene, which belongs to the NtrC family. Expression analysis of the *pcuCAXB* operon using a reporter gene fused to the promoter showed that only substrates of the pathway, such as *p*-cresol, *p*-hydroxybenzyl alcohol or *p*-hydroxybenzyl aldehyde, were effectors of PcuR. Neither toluene nor *p*-hydroxybenzoate was able to induce expression of the pathway[8]. The *pobA* gene codes for *p*-hydroxybenzoate hydroxylase, which converts *p*-hydroxybenzoate into protocatechuate, the substrate for ring fission[37, 132]. It has been shown that in this strain, expression of the *pobA1* gene is under the control of the divergently transcribed *pobR1* gene, which belongs to the AraC family. However, no data are available on the specific molecular mechanisms involved in this process.

5. OVERVIEW OF THE REGULATION OF THE TBU AND TOM PATHWAYS IN R. PICKETTII AND B. CEPACIA

Ralstonia pickettii PK01 (formerly *Pseudomonas pickettii* PK01) is able to grow on toluene, phenol and benzene as the sole carbon and energy source[98]. The pathway responsible for the degradation of these compounds to TCA cycle intermediates is called the toluene-3-monooxygenase pathway (also *tbu* pathway for *t*oluene *b*enzene *u*tilization) (Figure 1). The genes

that encode enzymes for the pathway are grouped in three operons. The *tbuA1UBVA2C* and *tbuT* operons encode the initial toluene-3-monooxygenase and the transcriptional activator TbuT[16]. The *tbuD* operon encodes phenol/cresol hydroxylase[72, 74], and the *tbuWEFGKIHJ* operon encodes enzymes of the *meta*-cleavage pathway for the conversion of catechol and methylcatechols to tricarboxylic acid cycle intermediates[73]. In addition, *tbuX* codes for a putative facilitator of toluene entry into the cell[66], and is located immediately downstream from *tbuT*. In turn, TbuT, a member of NtrC family of transcriptional activators, controls transcription of each of these operons in response to aromatic effector compounds[16]. TbuT is activated by aromatic effectors (toluene, benzene and ethyl benzene) and trichloroethylene. Expression of *tbuT* is self-regulated, as suggested by the finding that it is linked to the expression of the *tbuA1UBVA2C* operon by read-through transcription of *tbuT* from the toluene-3-monooxygenase promoter. As a result, transcription of *tbuT* is low when the toluene-3-monooxygenase operon is not induced and high when expression of *tbuA1UBVA2C* is induced by toluene. Thus, the toluene-3-monooxygenase promoter drives the cascade expression of both the toluene-3-monooxygenase operon and *tbuT*, resulting in a positive feedback circuit[16]. Upstream from the σ^{54}-dependent toluene-3-monooxygenase promoter (P_{tbuA1}), a DNA region with dyad symmetry may serve as the TbuT-binding site[16, 80]. Two additional regulatory genes, TbuS and TbuR, are located upstream from *tbuD*. In the absence of effectors, TbuS represses transcription of *tbuWEFGKIHJ*. The current view of the regulatory mechanism suggests that in the presence of the effectors phenol or *m*-cresol, these compounds interact with TbuS and the effector–TbuS complex acts as a transcription activator of *tbuWEFGKIHJ*. In addition, the effectors that interact with TbuR form complexes able to activate transcription of *tbuD*[98].

Burkholderia cepacia G4, formerly *Pseudomonas cepacia*, degrades toluene through a unique initial step that involves toluene-*ortho*-monooxygenase (TOM) (Figure 1). This strain also contains a catechol 2,3-dioxygenase for the *meta*-cleavage of methylcatechol. Due to its unique substrate specificity, the biochemistry of the TOM enzyme has been studied extensively. However, little is known about the genetics and regulation of the pathway. The genes responsible for this pathway are located in a 70–100 kb megaplasmid present in this strain. It has been shown that expression of the pathway is constitutive[119].

Burkholderia sp. strain JS150 contains a plasmid that carries the genes encoding for a toluene-2-monooxygenase clustered in the operon *tbmABCDEF* and its NtrC-like regulator coding gene *tmbR*. Additionally, a toluene-4-monooxygenase activity was assigned to an independent region of the same plasmid, which was shown to be regulated by TbmR[64b]. Finally, cross-activation between toluene-3-monooxygenase and toluene-2-monooxygenase by regulators TbuT and TbmR has been reported[80].

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